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Dissertation

Induced Resistance to Bacitracin and Penicillin in Cultures of *Staphylococcus aureus*

by

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Introduction

Antibiotics or antibiotic substances are products which can be extracted from a microorganism, and are antagonistic to the development or life of a microorganism or microorganisms other than the one producing it. In some instances antibiotics can be classified as chemotherapeutic agents since they fulfill the requirements of inhibiting or killing pathogenic microorganisms that have invaded the body and are more toxic to the pathogens than to the body tissues. In addition, a chemotherapeutic agent should be of known chemical composition; so most antibiotics are ruled out as chemotherapeutic agents due to this qualification. In general, however, biological products are not included as agents of chemotherapy.

The production of antibiotics by various organisms and the practice of antibiosis is considered to be a mechanism of preservation and survival among competing microbes of various species with the tendency to maintain equilibrium in a given microbial population. Often the addition of a new species to a particular environment which has been in a state of microbial equilibrium will result in the rapid destruction of the new species due to the strong antibiotic effect of the pre-

existing organisms. For example, if a pathogenic organism such as Salmonella typhosa is inoculated into fresh unsterilized soil, it will survive only a short time; but if the soil was previously sterilized to kill the antagonistic organisms, S. typhosa survives for a much longer period. This situation also occurs in water, sewage, garbage, and other natural environments containing many competing organisms. In some instances conditions may favor the survival and development of the microorganism that is added. In that case there will be an alteration in the original equilibrium and certain pre-existing species may be destroyed and eliminated from that environment. With few exceptions, human and animal pathogens are unable to survive for long periods in soil, water, garbage, etc., in part as a result of the phenomenon of antibiosis.

According to Waksman,[77] the antagonistic effects of one organism upon another were observed by many of the early microbiologists. In 1876 Tyndall first noted the struggles between mixed cultures of bacteria and Penicillium finding that in some cultures the former outgrew and eliminated the latter, and in other tubes the reverse was true. In 1877 Pasteur noticed that anthrax did not develop in susceptible animals that received simultaneous inoculations

of Bacillus anthracis and certain other bacteria. He stated

> "..... on peut introduire à profusion dans un animal la bactéridie charbonneuse sans que celui-ci contracte le charbon; il suffice qu'au liquide qui tient en suspension la bactéridie on ait associé en même temps des bactéries communes."

In 1879, De Bary remarked upon the observation that when two organisms are grown in the same medium, sooner or later one overcomes the other and even kills it.

There are several known methods for isolating antagonistic microorganisms from various natural sources, such as soil, sewage, water, garbage, etc.

The soil enrichment method consists of adding known living pathogenic bacteria to natural soil in a beaker and incubating. From time to time samples of the enriched soil are removed to test for the presence of organisms that are antagonistic to the added test organism.

A second method is the bacterial agar plate method. This consists of adding melted agar containing a suspension of the test organism to a series of petri plates, each having 1.0 ml of fresh or enriched soil, diluted from 1:100 to 1:10,000 times with sterile distilled water. The plates are then incubated and colonies of the soil organisms surrounded by zones of inhibition of the test bacteria are isolated and studied further for antagonistic activity.

The crowded plate method entails plating out field or garden soil in low dilutions to produce deliberate crowding by a large number of colonies. In this way potential antagonists that are normally present in the soil may manifest their presence by clear zones around the colonies.

The direct soil inoculation method consists of inoculating agar plates with the test organism and incubating for 24 to 48 hours. After good growth is obtained, particles of fresh or enriched soil are placed on the surface of the plate; if antagonistic organisms are present in the soil they will bring about killing or even lysis of the original culture.

A fifth method is the "forced antagonism" method by which one culture is continuously fed with another so that the former is forced to develop the capacity to destroy the first.

Antibiotics or extracts from these antagonistic organisms have been isolated from a variety of sources. They are by no means limited to products of microbial origin, as antibacterial substances have been isolated from higher plants (allicin from Allium sativum, inhibin from honey, tomatin from the tomato plant, etc.), from

animals,(erythrin from red blood cells, lacterin from milk, lysozyme from eggs, tears, and various organs),and from algae (chlorellin from species of Chlorella). These antibacterial products from higher plants and from animals are not antibiotics in the strict sense but have similar properties.

The earliest antibiotic to be tested for its therapeutic value was pyocyanase which was obtained by ether extraction of broth cultures of Pseudomonas aeruginosa. It was somewhat effective in the treatment of experimental anthrax. The crude product is extremely toxic, but several crystalline fractions have been prepared from the crude product and these less toxic derivatives retain their antibiotic activity, especially against Gram-positive organisms. The term "pyocyanase" is a misnomer since it does not act like an enzyme.

In addition to pyocyanase, Pseudomonas aeruginosa produces another distinct antibiotic, pyocyanine, which is the characteristic pigment of this species. Pyocyanine has been prepared synthetically, is strongly germicidal against a variety of bacteria especially Gram-positive organisms, but is too toxic for clinical use. Pyocyanine was said to be more effective against younger cells, whereas

pyocyanase exerts its strongest action on older cultures. Its mechanism of action as well as part of its toxicity is involved in its oxidation-reduction equilibrium with its colorless reduced form. It forms a reversible redox system and increases the oxygen consumption of both bacteria and red blood cells.

Another antibiotic of bacterial origin is tyrothricin which was isolated by Dubos in 1939 from an aerobic, Gram-positive, sporeforming soil bacillus, Bacillus brevis. Tyrothricin is composed of a mixture of polypeptides and can be separated into two crystalline components, gramicidin and tyrocidine, which are also polypeptides. The composition of tyrothricin is 10 to 20 per cent gramicidin, 40 to 60 per cent tyrocidine, and a residual portion containing substances that are similar to gramicidin and tyrocidine plus a small inactive fraction, which is partly lipoidal. Tyrothricin is soluble in alcohol and insoluble in water. It is active biologically chiefly against Gram-positive bacteria, but due to its toxicity, mainly a hemolytic action, it can be used clinically only for topical application and in body cavities such as sinuses.

Gramicidin, one of the components of tyrothricin, is a polypeptide soluble in ether and in alcohol, but

insoluble in water, and it is thermolabile. It has strong antibiotic properties both *in vitro* and *in vivo* mainly against Gram-positive organisms and *Neisseria*, but cannot be injected into the body due to its potent hemolytic properties. It can be used topically and in body cavities in man and other animals. Its mechanism of antibacterial action like that of pyocyanase is due to the increase in oxygen consumption of bacterial cells in the presence of gramicidin, that is, gramicidin stimulates the respiration of susceptible bacteria. The action of gramicidin is inhibited in the presence of serum and Gram-negative organisms. This blocking is considered to be due to the presence of phospholipids in the serum and in Gram-negative bacteria. In addition to increasing oxygen uptake of susceptible bacteria, it appears also to block uptake of phosphorus by these microorganisms. It is possible that gramicidin prevents the conjoined storage of carbohydrate and phosphorus in the bacterial cell.

The second component of tyrothricin, tyrocidine, is strongly bactericidal and even bacteriolytic to some organisms. Its chief action is against Gram-positive bacteria, but it also is effective against many Gram-negative bacteria. It is soluble in alcohol but insoluble in ether and is thermostable. Unlike gramicidin

tyrocidine has free amino groups and can combine with protein or partially hydrolyzed proteins. As a result of this characteristic, the antibacterial action of tyrocidine is markedly decreased in the presence of protein, peptone, and similar substances. In the presence of protein and related products, the predominant action of tyrothricin is due to the gramicidin. The ability to lower surface tension adequately explains both the bactericidal and bacteriolytic properties of this antibiotic. Tyrocidine is also prevented from being used parenterally by its hemolytic action. It differs from gramicidin, which produces a slow prolonged hemolysis, by producing a rapid, acute lysis of erythrocytes.

Gramicidin S (Soviet gramicidin) is produced from a Russian strain of Bacillus brevis. Its properties are more similar to tyrocidine than gramicidin. It is a thermostable crystalline polypeptide and can be autoclaved without appreciable loss of potency. Like tyrocidine and unlike gramicidin, it contains free amino and carboxyl groups and has been reported to be active against Gram-positive and certain Gram-negative organisms including Proteus vulgaris and Escherichia coli. It has been used effectively in the clinical treatment of infections in gunshot wounds of soft tissues, in severe burns, abscesses

of the abdominal wall, in pleural and peritoneal infections, and in anaerobic infections. The potency was not diminished in the presence of pus, it did not irritate the tissues, and had a favorable effect on regeneration and epithelization.

Other antibiotics extracted from bacterial sources include the seven relatively recent separate substances extracted from Bacillus subtilis. These are subtilin, subtilysin, endosubtilysin, bacitracin, bacillin, eumycin, and trypanotoxin. In this group only two show promise of taking their place among the family of clinically useful antibiotics. These two are subtilin and bacitracin.

Subtilin is obtained by extraction of cultures of **B.** subtilis with normal butanol. Best production is obtained by adding 0.3 per cent 1 (+) asparagin D plus iron, manganese, and copper to the basic medium. In general Gram-positive organisms and Neisseria are most susceptible in vitro. It is also active against Trypanosoma equiperdum and Entamoeba histolytica in vitro. It is not a highly stable compound and rapidly loses potency on storage. It has been shown to have high protective and therapeutic value in experimental infections with Staphylococcus, Streptococcus, pneumococcus, and anthrax

bacillus. Its mode of action is unknown although it has been found to lower surface tensions of water or alcohol to an equal extent as gramicidin. The general toxicity of subtilin is low.

Bacitracin, a polypeptide by all chemical tests, is a relatively nontoxic, highly active, stable (on refrigeration) antibiotic. It was originally extracted from B. subtilis by Johnson, Anker, and Meleney in 1945, and was found by them to have low toxicity when injected parenterally into mice, guinea pigs, or rabbits, or by subcutaneous, cutaneous, or conjunctival application in humans. Its antibacterial activity is chiefly against Gram-positive bacteria and Neisseria in vitro and has protected mice and guinea pigs against hemolytic streptococcus and certain clostridia in experimental infections by in vivo tests. Its pharmacological properties such as rate of absorption by various portals of entry, its blood levels and diffusibility into other body fluids and tissues, its acute and chronic toxicities, and its routes and rates of excretion have also been studied. It was determined that appreciable levels were attained rapidly by various parenteral routes, but that bacitracin was destroyed in the gastro-intestinal tract when given per os. The serum levels attained by intramuscular and subcutaneous injections were maintained at appreciable levels for con-

siderable periods, but intravenous injections gave high levels followed rapidly by a rapid drop. Excretion was largely in the urine. It did not penetrate the red blood cells nor did it diffuse readily into the spinal fluid. The acute and chronic toxicities were relatively low. Bacitracin is being produced on a pilot plant scale but the maximum yields are still only 40 units per mg. These, however, are only about one half as toxic as the previous maximum of 25 units per mg, indicating that the toxicity probably will decrease with greater purification.

A third antibiotic from B. subtilis, bacillin, was isolated in 1946 by Foster and Woodruff. Despite its potent antibacterial properties against a wide range of Gram-positive and Gram-negative organisms, its toxicity proved to be too great for clinical use. It is also inactivated by a wide variety of substances in nature.

The other antibiotic substances from B. subtilis are either of too great toxicity or too insoluble for practical use in the treatment of clinical disease. Some hope is seen for eumycin in the treatment of tuberculosis. It has strong antagonism against the tubercle bacillus and it has been suggested that its toxic factors may be separable from its antituberculosis properties.

Other prominent antibiotics from bacterial sources are the very recently isolated polymyxin from Bacillus polymyxa and aerosporin from Bacillus aerospora. These two are considered to be identical and are active against Gram-negative organisms. They have low toxicity for higher forms of life and microorganisms show little tendency to develop resistance to them.

A number of antibiotics have been produced by the actinomycetes, among which the most investigated ones are actinomycetin from Streptomyces albus, actinomycin from S. antibioticus, proactinomycin from Nocardia gardneri, streptothricin from S. lavendulae, and streptomycin from S. griseus.

Actinomycetin has some bad features, such as greatly diminished antibiotic activity in the presence of complex culture media, and the presence of the living Actinomyces is usually essential to produce death and lysis of the susceptible Gram-negative and Gram-positive bacteria.

Actinomycin is a highly potent antibiotic, antagonistic to all species of bacteria tested and many fungi, and is very stable to heat. It is, however, unsuitable for clinical purposes since it is only slightly soluble in

water and very toxic to animals.

Proactinomycin is very active against many Gram-positive bacteria and somewhat less active against some of the Gram-negative bacteria. It is soluble in water as well as some organic solvents, and is thermostable, but has limited therapeutic value due to its toxicity.

Streptothricin is far more valuable than the other antibiotics already extracted from Actinomycetes with the exception of streptomycin. It is soluble in water, thermostable, highly active against various Gram-negative and some Gram-positive bacteria, and is of limited toxicity to animals.

Streptomycin is the most investigated, most used, and probably the most therapeutically effective antibiotic isolated from the Actinomycetes. Its antibacterial spectrum is broader than that of streptothricin, being active against a wide variety of Gram-negative and Gram-positive bacteria, and it is less toxic than streptothricin. It is also very thermostable and highly soluble in water, In acid solution it is hydrolyzed into two inactive fractions, streptidine and streptobiosamine, a disaccharide. Although streptomycin is stable to heat, its activity can be destroyed by other mans, such as extremes of acidity or alkalinity (maximal activity is

at pH 8.). *In vivo*, the outstanding therapeutic uses have been against tularemia, meningitis caused by certain Gram-negative bacilli, especially *Hemophilus influenzae*. It has been effective in the treatment of urinary and respiratory infections caused by Gram-negative bacilli, such as *H. influenzae*, *Klebsiella pneumoniae*, *Escherichia coli*, and others. Definite clinical improvement has been observed with the use of streptomycin in certain forms of tuberculosis. The mechanism of action of streptomycin against susceptible organisms has not been explained. It differs from the action of penicillin, which is most effective against susceptible bacteria at the stage of most rapid cell division. A bad feature of streptomycin is the tendency for susceptible organisms to become increasingly resistant at a rapid rate generally without loss of virulence.

Other substances have been obtained from *S. griseus* in addition to streptomycin. Grisein is similar to streptomycin but has a narrower antibacterial spectrum, and actidione is a poor antibacterial substance, but is active against many yeasts, including *Cryptococcus neoformans*.

Many antibiotics have been isolated from various species of fungi, mainly among the genera *Penicillium* and *Aspergillus*. Prominent among these and among all anti-

biotics from all sources is penicillin which is produced from the molds, Penicillium notatum, and Penicillium chrysogenum. Four distinct penicillins, F, G, X, and K with different chemical formulae have been prepared. The most active and most commonly used clinically is penicillin G. Penicillin is effective against Gram-positive bacteria and the Neisseriae, but it has very little action against other Gram-negative bacteria except in very high concentrations. The toxicity of penicillin is extremely low. It is fairly stable on storage but is rapidly destroyed by heat, extreme acidity and extreme alkalinity. Its antibiotic activity is inhibited by ions of heavy metals, strong oxidizing agents, cysteine, and certain enzymes, such as clarase, taka-diastase, and penicillinase. Penicillins have little or no effect on resting cells but have a vigorous bactericidal effect on multiplying cells.

Other antibiotics produced by P. notatum are notatin (also known as penatin, E. coli factor and penicillin B) and notalysin. The action of notatin consists of the oxidation of glucose to gluconic acid with the production of hydrogen peroxide, and the antibacterial action is due to the hydrogen peroxide.

Another antibiotic from molds has been isolated from several species, and as a result, has many names. It is variously known as patulin, patulum, claviforme, claviformin, expansin, clavatin, and clavacin. It is effective against both Gram-positive and Gram-negative bacteria as well as many fungi. Its toxicity, however, is too great for clinical use.

Citrinin, obtained from Penicillium citrinum and Aspergillus candidus, is effective against Gram-positive bacteria with little or no effect on Gram-negative organisms. It has limited toxicity for animals.

There are several antibiotics produced by various species of Aspergillus. Four distinct antibiotics can be isolated from A. fumigatus; they are fumigatin, gliotoxin (also produced by species of Trichoderma, and gliocladium), spinulosin (also extracted from A. spinulosum), and fumigacin (known also as helvolic acid).

Fumigatin shows practically no action on Gram-negative and only limited action on Gram-positive bacteria, and is therefore, not an effective antibiotic.

Gliotoxin is active against various bacteria and fungi but is toxic to animals.

Spinulosin is a very weak antibiotic agent.

Fumigacin is active against Gram-positive bacteria, and although less toxic than many other antibiotics, it is too toxic for therapeutic use.

Some of the other better known antibiotics from aspergillar sources are aspergillic acid from A. flavus and aspergillin from A. niger. Aspergillic acid is active chiefly against Gram-positive bacteria, but also is active against many Gram-negative organisms. It is too toxic for clinical use. Aspergillin is effective against both Gram-positive and Gram-negative bacteria, is nontoxic, but is apparently water insoluble.

With the continued use and study of the various antibiotics the problem of resistant strains, due to repeated exposures to sublethal doses of the drug, becomes increasingly prominent. This problem was particularly evident with the use of streptomycin, since many originally susceptible strains developed a rapid and permanent rise in resistance to it without an accompanying decrease in virulence. This resistance was in some instances so rapid and so permanent that some strains became not only resistant to streptomycin but actually dependent upon its presence in the medium as an essential or accessory growth factor.[33,39]

For penicillin the problem of resistance was not quite as serious, since the resistance does not ordinarily rise as rapidly; and simultaneous with the increase, there is many times a drop in virulence. There are, however, many strains among susceptible species which are naturally resistant, and susceptible strains may become resistant during exposure to sublethal doses without loss of virulence.

Theories have been advanced regarding the mechanism of the development of resistance, and these have been summarized by Waksman[16] into three main mechanisms of resistance among bacteria. These are adaptive mechanism (A), selective mechanism (S), and mutating mechanism (M). The adaptive mechanism consists of the production by the organisms of new enzyme systems or new metabolic mechanisms to replace those interfered with by the presence of the antibiotic or by some other related phenomenon, such as the production of inactivators of the antibiotic, the outstanding example of this being penicillinase. The selective mechanism consists of the elimination of susceptible cells from a given bacterial population by the antibiotic, and as a result the more resistant individual cells survive and dominate the bacterial population. The mutating mechanism, as the name

implies, consists of the arising of resistant mutations from a susceptible parent strain.

The overcoming of resistance to antibiotics is one of the major problems confronting workers in various fields of science. Since one possible solution to this problem is the use of more than one antibiotic either simultaneously or successively, it was considered of interest to study the use of resistance to a new antibiotic, bacitracin, as compared with penicillin. This study is concerned with the rise of resistance of Staphylococcus aureus (Micrococcus aureus) following exposure to penicillin and bacitracin, the existence or nonexistence of cross resistance, and the decrease in resistance following removal of the resistant strains from the antibiotic.

HISTORY

Nicolle (1907) [43] revealed that cultures of Bacillus subtilis were antagonistic toward some of the Clostridia, and that filtrates of B. subtilis were active against many bacteria such as Pneumococcus, Glanders bacillus, Anthrax bacillus, Typhoid bacillus, Shigella, Salmonella suipestifer, Staphylococci, Vibrio cholerae, Bacillus pestis, and others.

Pringsheim (1920) [47] discussed the antibiosis of various organisms showing that many bacteria possessed the ability to inhibit other bacteria.

Rosenthal (1925) [57] found that Bacillus mesentericus had a lytic activity on various thermophilic bacteria, for example staphylococci, Salmonella typhosa, and Vibrio cholerae both in broth and on gelatin plates.

Rosenthal (1925) [55] also showed that Tyrothrix scaber was active against Vibrio cholerae, and in the same year [56] found it to be active against other thermophilic bacteria as well as having a lytic effect on these organisms.

Van Canneyt (1926) [74] revealed that Bacillus subtilis overgrew Mycobacterium tuberculosis when the latter organism had been previously cultured on a potato medium, and lessened

the growth of the tubercle bacillus so that it was almost gone in five to seven weeks.

Rakieten, Rakieten, and Duff (1936) [48] reported that several strains of bacilli in the subtilis group were able to adsorb and inactivate Staphylococcus bacteriophage in 18 hours at 35 C, and once inactivated the phage could not be freed by the addition of susceptible staphylococci or by lysis of the adsorbing bacilli. The possible mechanism of this action was discussed. In addition these workers were able to inactivate a virulent strain of vesicular stomatitis by contact with Bacillus subtilis for 15 hours at 35 C.

Rosenthal (1941) [58] studied the inhibitory and lytic action of 31 strains of the subtilis-mesentericus group against Gram-positive and Gram-negative organisms. Of the 31 strains, 7 were inactive, 6 were active against Gram-positive bacteria only, and the remaining 18 were active against both. The living Gram-positive bacteria were more susceptible to the inhibitory action than the living Gram-negative bacteria, while the dead Gram-negative organisms were more susceptible to the lytic activity than the dead Gram-positive organisms.

Heilman and Herrell (1941) [22] showed that gramicidin was strongly hemolytic to rabbits'or sheep's erythrocytes whether in tissue culture, on a blood plate, or in blood broth.

In 1941 Heilman and Herrell[23] also studied the mode of action of gramicidin and tyrocidin. The action of tyrocidin was concluded to be a lowering of surface tension in the manner of a detergent. Gramicidin was considered to have a similar action. It is now known that at least part of the action of gramicidin is effected by increasing the ocygen consumption of the organisms.

Robinson and Molitor (1942) [54] investigated the toxicity of gramicidin, tyrocidin, and tyrothricin in rats and mice and other experimental animals. In rats and mice oral doses were non-toxic up to 1 g per kg, possibly due to its insolubility. Intravenous doses of gramicidin were lethal in 1 to 30 mg per kg, of tyrothricin 2.5 mg per kg, and of tyrocidin 15 mg per kg. These observations were carried for from 1 to 4 days. Intraabdominally tyrothricin caused death at 30 mg per kg, whereas gramicidin and tyrocidin killed at 40 mg per kg. All three drugs showed greater toxicity by both intravenous and intraabdominal routes when observations were recorded up to seven days.

Lewis, Dimick, Feustel, Fevold, Olcott, and Frankel-conrat (1945) [35] modified gramicidin by mixing 10 parts of 5 per cent solution of gramicidin in 95 per cent alcohol with 1 part of 1 normal sodium hydroxide and 5 parts of

commercial 40 per cent formaldehyde and maintaining at 53 C for two days. The formaldehyde-modified gramicidin was less than one-twentieth as toxic by intraabdominal injections into rats and less than one-tenth as hemolytic as untreated gramicidin. It still maintained its original antibacterial potency.

Rees and Reardon (1944) [53] showed that penicillin in a concentration of 100 units per ml could be used to eliminate Streptococcus hemolyticus as a contaminant from N I H 563 medium in the endeavor to culture Entamoeba histolytica, and that it was useful to eliminate bacteria from cultures of Trichomonas vaginalis.

Van Dyke (1944) [75] studied some pharmacological properties of crystalline penicillin G and determined that: (1) 0.15 M sodium penicillin produced no hemolysis of washed rabbit erythrocytes after 3 hours at 37 C, but produced some changes in the red blood cells after 17 hours; (2) there was no appreciable adsorption of sodium penicillin on red blood cells after two hours' incubation at 37 C of erythrocytes in a medium containing sodium penicillin G; (3) no changes in oxygen consumption of yeast cells, duck red blood cells, and rat liver slices, as a result of exposure to penicillin for over two hours; (4) high concentrations of sodium penicillin caused moderate reversible

reduction in amplitude of beat of frog's heart but had no effect on the rate; (5) it had no effect on isolated guinea pig ileum or uterus; and (6) extremely high intravenous concentrations in mice caused only very slight, transient toxic effects.

Bondi and Dietz (1944) [7] reported a method of determining penicillinase production by a test organism. The organism is grown for 4 days in broth and mixed with penicillin in proper dilutions and amounts. The mixture is then placed in a penicylinder cup on agar plates previously seeded with a penicillin sensitive organism. If there is no zone of inhibition and a control cup shows inhibition of the penicillin sensitive organism, penicillinase was produced.

Bondi and Dietz (1945) [9] studied 115 strains of Staphylococcus, not all pathogenic, hemolytic, or pigmented, isolated from patients. The 115 strains were assayed for penicillin sensitivity and then penicillinase determinations were made. Strains that were inhibited by 0.15 units of penicillin per ml or less (99 of the 115 strains) did not produce penicillinase, whereas strains (the remaining 16) that required more than 0.15 units per ml for inhibition did produce penicillinase.

Perlstein and Liebmann (1945) [46] produced an anti-penicillinase immune serum by injecting rabbits with penicillinase, and this serum protected penicillin (1000 units) *in vitro* from up to 50 units of penicillinase for one hour, whereas normal serum protected against only 3 units of penicillinase under similar conditions.

Gots (1945) [18] developed a simplified method of determining penicillinase production by an organism. The method consists of inoculating a penicillin-agar medium with a penicillin sensitive organism, and then streaking the test organism over the surface of the plate after it has hardened. The test organism is streaked from the center out like the spokes of a wheel, and if the penicillin sensitive organism grows in the vicinity of the streak, it indicates penicillinase was produced and diffused into the medium. The width of the zone of growth by the penicillin sensitive organism is a rough estimation of the amount of penicillinase produced.

Benedict, Schmidt, and Coghill (1945) [5] tested several enzymes for penicillinase activity and found only two, concentrated pumpkin protease and high diastatic malt syrup, that had activity. Also numerous organisms in the general *Serratia*, *Escherichia*, *Aerobacter*, *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Proteus*, and *Bacillus* were

tested for penicillinase production and were found to produce only a weak exocellular penicillinase. The optimal pH of penicillinase was reported as 6.5 to 7.0 at 25 C. The destruction of penicillin was rapid, more than half of 60,000 units being destroyed in 8 minutes.

Katznelson (1942) [29] found that Bacillus subtilis produced a thermostable substance active against Rhizoctonia soloni, and Humfeld and Feustel (1943) produced antibacterial extracts from two species of Bacillus subtilis grown on asparagus juice medium.

Jansen and Hirschman (1944) [26] reported on the extraction of a substance (subtilin) from cultures of Bacillus subtilis. A synthetic medium was employed to eliminate excessive organic substances, and four transplants were made for best subtilin production. The three preliminary subcultures were incubated at 35 C for 24 hours, and the final transplant was made into a flask with the desired volume of medium using a 4 per cent inoculum. The final subculture was incubated, and on the 3rd and 6th days tests were made for the presence of subtilin. To test for subtilin a few drops of 1.0 per cent alcoholic solution of ferric chloride were added to 5.0 ml of the culture. A deep blue color developed if there was high subtilin activity in the culture, and no color developed with little subtilin

activity. Best production was obtained by addition of 0.3 per cent l (-/-) asparagine D plus iron, manganese, and copper to the basic medium. A crude dry form of subtilin was extracted and purified. Antibacterial properties against test organisms and in standard test media were performed using Staphylococcus aureus (FDA #209) and Micrococcus conglomeratus (Merck "M.Y.") in nutrient broth and Lactobacillus casei in a yeast extract-glucose broth. Studies on stability showed it to be relatively unstable with rapid loss of activity on storage. There was a far greater loss of activity if the material was stored (either in dry form or in solution) in the light than if it was stored in the dark.

Ramon and Richou (1945) [50] studied the action of a substance extracted from Bacillus subtilis, which was termed subtilin, on diphtheria, staphylococcus, and tetanus toxins. These toxins were either modified or destroyed by exposure to subtilin.

Olivier, de Saint-Rat, Bonet-Maury, and Blanchon (1945)[44] studied the effect of ingestion of cultures of Bacillus subtilis in the treatment of diarrheas due to bacteria, and obtained good results. Treatment of cystitis and pyelonephritis due to colon bacillus by ingestion or bladder injection of B. subtilis cultures also gave favorable results. The mechanism was considered

to be due to the replacement of the pathogen by B. subtilis since it grew more rapidly, and this was followed by the subsequent disappearance of B. subtilis as well.

Ramon, Richou, and Ramon (1946) [51] extracted and partially purified a substance from Bacillus subtilis which they showed by a tube method to be antagonistic in vitro to the diphtheria bacillus, anthrax bacillus, and cocco-bacillus of pseudotuberculosis.

Salle and Jann (1945) [59] employed a cup plate or disc method to determine the antibacterial spectrum of subtilin in dilutions running from 1:1000 to 1:10,000,000. They considered as susceptible those organisms that were inhibited in dilutions of 1:1000 or higher and as resistant organisms those that were not inhibited at 1:1000. In general Gram-positive bacteria were susceptible while Gram-negative bacteria were resistant.

Anderson, Villela, Hansen, and Reed (1946) [1] found that subtilin lowered the surface tensions of water or alcohol to an equal extent as gramididin, but unlike gramicidin it had no effect on red blood cells. Studies on certain protozoa revealed that it produced cytolysis of Trypanosoma equiperdum in vitro but was ineffective in protecting mice from an infection by T. equiperdum;

it was inactive *in vitro* and *in vivo* against *Leishmania donovani*; it was very active against *Entamoeba histolytica in vitro* and also cleared one *Macacus rhesus* of infection for 3 weeks following the administration of 1 g per kg total oral dosage of subtilin in 10 days; and it was also active against *Lactobacillus plantarum in vitro*, particularly in the presence of para-aminobenzoic acid which enhanced this action of subtilin. Toxicity studies in mice showed the intravenous L D_{50} to be 60 $\pm$ 3 mg per kg as compared to gramicidin whose L D_{50} is 1.5 mg per kg. Subcutaneous and intragastric L D_{50} of subtilin were 670 $\pm$ 30 mg per kg and 5 g per kg respectively.

Salle and Jann (1946) [60] determined the toxicity index of subtilin as 0.05, using the highest dilution of subtilin that killed embryonic tissue after 10 minutes' exposure at 37 C as the numerator, and using the highest dilution killing *Staphylococcus aureus* (F.D.A. strain) under the same conditions as the denominator. They also defined a unit of subtilin as the amount present in 1.0 ml of the highest dilution (expressed in mg) capable of killing *Staphylococcus aureus* (F.D.A. strain) in 10 minutes at 37 C.

Salle and Jann (1946) [61] were able to protect and cure mice from experimental infections of lethal doses of pneumococcus Type III by injections of 0.5 units every four

hours or less frequently for two to three days. There were no observable toxic reactions from the subtilin.

Salle and Jann (1946)[62] treated guinea pigs that were experimentally infected with Bacillus anthracis by intraabdominal injections of subtilin and were able to prolong the lives of the guinea pigs and possibly cure them.

Salle and Jann (1946)[63] protected and cured mice of experimental infections of Streptococcus pyogenes by injections of one unit of subtilin every 3 hours, later increasing to two units at longer intervals, for approximately 30 hours.

Wong, Hambly, and Anderson (1947)[79] used a modified Dubos and Davis Medium for the demonstration of the antibiotic activity of subtilin against Mycobacterium tuberculosis. Using this medium control cultures grew in three days, while subtilin inhibited the growth of M. tuberculosis as follows: final concentrations of subtilin from 1:20,000 through 1:400,000 inhibited growth for 12 days; 1:800,000 inhibited growth for 4 days, and 1:1,600,000 inhibited growth for 3 days, so that there was growth on the fourth day after inoculation. Material taken from these cultures for guinea pig inoculation

revealed that a dilution of 1:20,000 prevented tubercle bacillus infection, 1:40,000 and 1:80,000 sometimes prevented infection, but higher dilutions did not prevent infection.

Salle (1947)[64] discussed subtilin in regards to methods of preparation, physical and chemical properties, including stability, antibacterial spectrum, toxicity, and *in vivo* activity.

Vallee (1945) [73] studied the action of extracts of *Bacillus subtilis* (these extracts were termed subtilysin) against *Vibrio septique* and *Bacillus oedematiens*. He found that by mixing the extracts with the cultures for short periods of time prior to injection into guinea pigs did not protect the animals, but when subtilysin was incubated with cultures of *Vibrio septique* or *B. oedematiens* for longer periods the survival rate of the guinea pigs was up to five times as long as the controls.

Buggs, Bronstein, Hirshfeld, and Pilling (1946)[10] found a heat labile factor in human sera that was able to inhibit *B. subtilis* in varying dilutions up to 1 to 32. These same were tested against *Staphylococcus aureus*, but none were inhibitory for that organism.

Foster and Woodruff (1946)[15] isolated bacillin, a new antibiotic from *Bacillus subtilis*, and distinguished

this antibiotic from tyrothricin, subtilin, simplexin, and bacitracin. Bacillin revealed antibacterial properties against a wide spectrum of Gram-positive and Gram-negative bacteria, but its toxicity in mice proved to be greater than its protective capacity against the pneumococcus.

Foster and Woodruff (1946)[16] showed that antibacillin, a naturally occurring inhibitor of bacillin, was present in a wide variety of organic material, such as brain-heart infusion, tryptone, rabbit blood, rabbit serum, many bacteria, yeasts, molds, and actinomycetes.

Burdon and Johnson (1947)[11] reported on the present status of eumycin, another antibiotic isolated from Bacillus subtilis. It was stated to be antagonistic to pathogenic fungi and to the related actinomycetes, as well as members of the genera Mycobacterium and Corynebacterium. Methods of production and extraction, chemical and physical properties, and toxicity were discussed. It was considered too toxic for clinical use although some hope was held for its use against tuberculosis.

Johnson, Anker, and Meleney (1945)[27] extracted a substance unlike subtilin from Bacillus subtilis and termed it bacitracin. The physical, chemical, pharmacological and antibiotic properties were investigated.

It was reported to have low toxicity when injected subcutaneously or intraabdominally into mice or guinea pigs, intravenously into rabbits, or by subcutaneous, cutaneous, or conjunctival application in humans. A standard unit was designated and an *in vitro* antibacterial spectrum was determined. The antibacterial tests in broth revealed considerable activity against hemolytic streptococci of Groups A, B, C, and G, and some activity against *Streptococcus* Group D, *Staphylococcus aureus*, pneumococcus Types I, II, and III, and some of the war wound anaerobic bacilli. By the agar plate penicylinder method gonococci and meningococci showed a zone of inhibition, but certain Gram-negative bacilli were not inhibited. *In vivo* tests in mice and guinea pigs showed considerable protection against hemolytic streptococci and *Clostridium perfringens* or *Clostridium septicum*. Local application to streptococcal or staphylococcal infections in a small number of human cases gave results comparable to those with locally applied penicillin.

Scudi and Antopol (1947)[66] studied the acute and chronic toxicities of bacitracin and revealed that the L D_{50} in mice was approximately 200-300 mg per kg intravenously or intraabdominally, the subcutaneous L D_{50} was about 2500 mg per kg, and the oral L D_{50} was approximately 7.5 g per kg. The L D_{50} was about the same for active and

inactivated bacitracin. The toxicity was found to be very low when small doses were given over an extended period of time, despite the fact that the total dosage was well over the L D_{50}.

Scudi, Clift, and Krueger (1947)[67] investigated the absorption and excretion of bacitracin in the dog by administrating the antibiotic by various routes and taking blood, urine, and feces specimens as assay materials for bacitracin at various intervals. The results showed that: (1) after oral administration no detectable bacitracin could be found in the serum or urine and only 5 per cent could be recovered in the feces indicating destruction of the antibiotic in the gastro-intestinal tract; (2) after a single subcutaneous injection maximal serum concentrations occurred in one hour and significant amounts were still present after 7 to 8 hours; (3) after intramuscular injection higher concentrations were obtained than those after a subcutaneous injection, and the duration of appreciable levels was about the same; (4) after an intravenous injection very high serum levels were achieved rapidly (as high as 15 units per ml) followed by a rapid drop; (5) no toxic effects were observed in the oral, subcutaneous, or intramuscular experiments, but transient slightly toxic effects were noted after high concentrations were

administered intravenously at a rapid rate; and (6) bacitracin did not penetrate the red blood cells nor freely enter the spinal fluid, and recovery in the urine was variable but generally increased in direct ratio to the dose administered.

Hoff, Stanley, and Bennett (1947)[25] described a cup plate method for the assay of bacitracin, the best results being obtained with Micrococcus flavus as the test organism. An eleven mm diameter zone was noted with 0.1 units per ml and a 21 mm zone with 4.0 units per ml.

Goorley (1947)[17] investigated some of the chemical and physical properties of bacitracin in an attempt to produce it on a pilot plant scale. Preparations were being made with a purity of about 30 to 40 units per mg. These preparations had an M L D for 20 g mice of about 300 units when injected intraabdominally, were completely soluble in water, and had only a slight pressor effect when 1000 units per kg were injected intravenously into dogs. The material containing 40 units per mg was about one half as toxic as the previous material containing 25 units per mg. Solubilities, methods of precipitating and purifying, and chemical tests (indicating that it is a polypeptide) were discussed.

Johnson, Anker, Scudi, and Goldberg (1947)[28] showed that smooth colonies of Bacillus subtilis yielded little or no bacitracin while rough strains yielded up to 4 to 8 units per ml of broth. Some substrains gave higher yields than the parent strains. Studies were made on the method of extraction and purification, and of the stability of the purified product under various conditions. In vivo studies revealed that subcutaneous injections of bacitracin were highly effective in the treatment of mice previously infected with 10,000 MLD of hemolytic streptococcus or pneumococcus Types I and II. Acute and chronic toxicity studies and absorption and excretion studies were also discussed.

Meleney and Johnson (1947)[38] observed the effects of local application of bacitracin to 100 cases of surgical infections, such as furuncles, deep and superficial abscesses, carbuncles, infected sebaceous cysts or wounds, ulcers in various locations, styes, and impetigo, and found that the majority of these cases gave either excellent or good results.

Hoff, Bennett, and Stanley (1947)[24] described a cylinder cup method for the assay of bacitracin.

Scudi, Coret, and Antopol (1947)[68] studied the toxic effects of intramuscular injections of commercial

bacitracin in dogs and monkeys for 4 weeks and 5 to 6 weeks respectively. There were practically no changes observed in the blood and urine of dogs during and at the end of the course, while there were some slight changes noted in the blood and urine of the monkeys. Autopsies revealed no macroscopic and slight microscopic changes in certain organs of the dogs, but there were definite gross and histological alterations in the monkeys.

Eagle and Fleischman (1948)[13] studied the relative antisyphilitic activity of penicillins F, G, K, and X, and bacitracin. They found that penicillin G far surpassed the other penicillins as well as bacitracin in the inhibition of the Reiter strain of Treponema in vitro, in the cure of an established syphilitic infection in rabbits, and in the abortion of a syphilitic infection during the incubation period.

Eagle, Musselman, and Fleischman (1948)[14] revealed that subtilin was far less active than either bacitracin or penicillin in producing either complete or partial inhibition of the Reiter strain of Treponema. They noted that by increasing the concentration of bacitracin they were able to cause a more rapid death of the Reiter strain. Bacitracin was far more effective than subtilin in the

treatment of experimentally produced syphilitic chancres in rabbits.

Against Streptococcus pyogenes (C-203) subtilin, however, was nearly as active as penicillin, which was 20 times as active as bacitracin.

Anker, Johnson, Goldberg, and Meleney (1948)[2] reported on the production, concentration, and purification of bacitracin. The maximum purity is still a crude product of 30 to 40 units per mg. They also reported on various chemical properties of the crude product.

Bond and Nook (1948)[6] described a penicylinder cup plate method for determining the concentration of bacitracin in body fluids; it was reported to be sensitive to 0.02 units per ml.

Rammelkamp and Maxon (1942)[49] showed that strains of Staphylococcus aureus that were originally highly susceptible to penicillin (most were inhibited between 0.02 units and 0.08 units per ml) developed increasing resistance after repeated contact with sublethal doses of penicillin. One strain developed a 64-fold resistance in 54 days. This rise in resistance by contact with sublethal doses occurred both in vitro and in vivo.

Schnitzer, Camagni, and Buck (1943)[65] found that small colony variants (g-forms) of Staphylococcus albus developed resistance to penicillin, whereas the S-form was still susceptible. This was, however, shown to be a non-specific drug resistance as these organisms also became resistant to other chemicals.

McKee and Houck (1943)[37] showed that although three strains of Staphylococcus developed resistance to penicillin of 1000, 4000, and 6000 times, the maximum resistance obtainable by three strains of Pneumococcus and a strain of Streptococcus pyogenes was 30 times the original. In addition there were indications that the strains which became resistant simultaneously lose virulence and this seemed to be of a permanent character.

Bondi and Dietz (1944)[8] also investigated strains of Staphylococcus aureus, Salmonella typhosa, and Proteus vulgaris that were made more resistant to penicillin and found that these organisms did not produce penicillinase, but were resistant due to some other mechanism.

Kirby (1944)[30] isolated 7 strains of staphylococci that were naturally resistant to penicillin and each of these 7 strains produced penicillinase.

Demerec (1945)[12] produced penicillin resistant

Staphylococci and this resistance was maintained by keeping the culture in the refrigerator on an agar slant for three months. A discussion of the origin of resistance was also included with the conclusion that it was due to development of mutants.

Todd and Turner (1945)[71] induced penicillin resistance by two strains of Staphylococcus aureus by cultivating the organisms in increasing concentrations of penicillin, and returned them nearly to their original susceptibility by transfers in penicillin-free broth.

Miller and Bohnhoff (1945)[41] grew gonococci in increasing concentrations of penicillin and raised the end point from 0.02 units per ml to 21 units per ml. A special technique of alternating the subcultures in penicillin agar and penicillin-free agar was employed to attain this resistance. By constant exposure to penicillin the gonococci were able to reach a maximal resistance of only 7.7 units per ml.

Todd, Turner, and Drew (1945)[72] increased the resistance of Staphylococcus A to penicillin by repeated subcultures in penicillin broth; 250-fold after 21 cultures, and 5,000-fold after 75 subcultures. The Oxford H strain of Staphylococcus aureus was 3000 times as resistant after 27 transfers and 5000 times as resistant after 75 transfers. Both strain rapidly lost this acquired

resistance with subcultivation in plain broth.

Using hemolytic streptococci as the test organism, the resistance was five times the original after 35 subcultures, and four times the original after 78 subcultures. The drop to original sensitivity was slow and required 76 subcultures in plain broth. There was considerable up and down variation in end points in both the rise and fall of resistance.

After 46 subcultures pneumococcus Type II became 26 times as resistant as originally and remained the same after 114 subcultures in penicillin blood broth. The penicillin fastness was maintained after 82 subcultures without penicillin, indicating a permanent fastness of pneumococcus to penicillin.

Miller and Bohnhoff (1945)[42] also studied the development of penicillin resistance by the meningococcus, and the simultaneous effect on virulence. With seven strains ranging in natural resistance from 0.1 to 0.5 units per ml, 6 strains reached a resistance up to 5.0 units per ml with no loss of virulence, while the seventh strain reached a resistance of 41 units per ml, with a complete loss of virulence at the resistance of 18 units per ml, but with no loss of virulence up to 14 units per ml.

Luria (1946)[36] utilized a pour plate method for determining the development of resistance of Staphylococcus

to penicillin. Dilutions of penicillin from 0 to 0.1 units per ml were utilized in the pour plates. The number of colonies gradually diminished from the plates containing no penicillin to no colonies in the plates containing 0.1 units per ml. Colonies from the highest concentration of penicillin showing growth were used for a second serial dilution pour agar test and these cultures showed increased resistance over the original cultures. Repeated such tests gave rising resistances. Some strains required heavy inocula to get gtowth, indicating the possibility that penicillinase was produced and sufficient inoculum was essential so that enough penicillinase to inactivate the penicillin could be transferred.

Graessle and Frost (1946)[19] studied 6 strains of staphylococci isolated from different sources for development of resistance to penicillin and streptomycin and other characteristics associated with resistance. They found that all strains become resistant when exposed to the antibiotics, that resistance was lost after transferring daily for seven days in nutrient broth or in some instances by storage or dry ice (for penicillin only). Resistance developed to penicillin in some cases showed slight cross resistance to streptomycin, but the reverse did not occur. The organisms developing resistance to

penicillin showed pleomorphism, slowing of metabolic activities, and loss of pigmentation; comparable effects were not found for streptomycin resistance except for an occasional loss of pigmentation and a slowing in rate of fermentation of sugars, but this was reversible after removal of the organisms from streptomycin for a sufficient period of time.

Yegian, Budd, and Middlebrook (1946)[80] studied biologic changes in a sulfathiazole resistant strain of Mycobacterium ranae. This strain, which was 200 times as resistant as the parent strain, maintained its resistance after 35 transfers in plain Long's Medium during a 10-month period; it showed cross resistance to sulfanilamide, sulfapyridine, and sulfadiazine. There were permanent color changes produced in the medium by the resistant organism as well as an increase in granularity of the pellicle. Also the resistant strain grew at a rate approximately one half that of the parent strain. There were, however, no changes in antigenicity or morphology as a result of the increased resistance. One observation was that there was a greater susceptibility to antibacterial agents such as oxalic acid, nitric acid, copper sulfate, phenol, etc., simultaneous with the rise of resistance to sulfathiazole; this was explained by the slower rate of growth.

Klein (1947)[31] tested 8 strains of staphylococci and streptococci for the development of strains resistant to penicillin and streptomycin. The end point of penicillin and streptomycin inhibiting the strains was first determined, and then varying concentrations of each antibiotic far in excess of the end point were used for isolating resistant organisms. The method consisted of inoculating 100 tubes of each concentration of antibiotic, and recording the number of positive cultures. Of the 8 strains tested in this manner with penicillin, none developed resistant variants with a high degree of resistance. Six strains were tested with streptomycin and a total of 57 variants with resistance to high concentrations developed.

Silver and Kempe (1947)[70] studied the mechanism of development of resistance to streptomycin using Escherichia coli and Aerobacter aerogenes as test organisms. Their experiments gave indications that a single strain when exposed to the antibiotic included variants with varying degrees of resistance, from extremely susceptible to highly resistant strains, and when the susceptible variants were inhibited, the more resistant variants grew out.

Seligmann and Wassermann (1947)[69] induced resistance to streptomycin by various organisms (Pseudomonas aeruginosa, Staphylococcus aureus, Serratia marcescens, and several

strains of Salmonella) both *in vitro* and *in vivo* and observed changes in biologic characteristics such as loss of pigment production in some cases, permanent resistance, and a slower rate of growth.

Waksman (1947)[76] discussed the development of bacterial resistance to streptomycin favoring the selective mechanism (S) as the explanation of rise in resistance.

Miller and Bohnhoff (1947)[40] studied 96 strains of meningococcus and found that none were naturally resistant to penicillin, but resistance was developed rapidly by repeated subculture in increasing concentrations of penicillin, particularly if the inocula were heavy. Virulence decreased with the rise of resistance but was readily restored by a few mouse passages. *In vivo* resistance increased correspondingly with *in vitro* resistance. There were slight, temporary morphological changes. Attempts to demonstrate penicillinase and antigenic differences between parent and resistant strains failed.

Kushnick, Randles, Gray, and Birkeland (1947)[33] found that strains of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* which became resistant to 1000,8000, and 1000 micrograms per ml of streptomycin respectively grew better and more rapidly in the presence of streptomycin. A variant of *B. subtilis* would not grow in the absence of streptomycin.

Weinstein and Tsao (1947)[78] induced resistance to penicillin by several strains of Streptococcus pyogenes from 4 to 32 times the original in 41 transfers. The resistance returned to normal after 37 subcultures in plain broth.

Klimek, Cavallito, and Bailey (1948)[32] studied the rises of resistance by Staphylococcus aureus to various antibiotics. The development of resistance to penicillin and the active principle of Asarum canadense was rapid with fluctuations, and there were accompanying morphological changes. Streptomycin-resistance increased very rapidly but showed no morphological changes. There were moderate increases in resistance to gliotoxin and pyocyanin. Those that developed resistance only slightly were the active principles of Arctium minus and Allium sativum. Mercuric chloride and aspergillic acid yielded no significant rise in resistance. In general there was a rapid loss of resistance to penicillin and gliotoxin, only a moderate reversal to streptomycin, and maintenance of resistance by Allium sativum and pyocyanin. There was a slight cross resistance to streptomycin in strains highly resistant to penicillin and slight cross penicillin resistance in streptomycin-resistant cultures.

Bellamy and Klimek (1948)[4] revealed that variants of Staphylococcus aureus which became highly resistant (60,000-fold) to penicillin changed their gram staining properties, developed cocc-bacillus forms, produced penicillinase, although penicillinase was not produced in the same strain at a lower resistance level, showed changes in fermentation reactions, and no longer required nicotinamide as an accessory growth factor.

Bellamy and Klimek (1948)[3] revealed that strains of Staphylococcus aureus which became resistant to penicillin grew at about one-half the rate of the sensitive parent strain aërobically and showed no appreciable growth anaërobically. They also pointed out that organisms such as Clostridium perfringens, Streptococcus fecalis, and Streptococcus mastitidis that depended on anaërobic processes for their energy supply will not develop appreciable resistance to penicillin.

Paine and Finland (1948)[45] obtained streptomycin-resistant and streptomycin-dependent strains of Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Proteus morgani by a single exposure of large numbers of "sensitive" organisms to high concentrations of streptomycin. The resistant and dependent strains represented only a very small proportion of the total number of

organisms that had been exposed to the drug.

It was noted for a particular strain that the sensitive and dependent cultures were inhibited at approximately the same concentrations. In other words, the dependent strain would cease to grow in media containing streptomycin in concentrations less than a given amount, while the sensitive strain would be inhibited in concentrations above that amount.

The mechanism was explained by the concept of the action of antibacterial agents as metabolite antagonists. Thus, in sensitive strains streptomycin can be considered as interfering with some essential metabolite or metabolic process, while in dependent strains, streptomycin serves as an essential metabolite or growth factor. Resistant strains may be indifferent to the presence or absence of streptomycin.

EXPERIMENTAL WORK

I. Methods and Materials

A. Test Organisms

Four strains of Staphylococcus aureus were employed in this study. The strains employed were the FDA strain #209, the Oxford strain obtained from the American Type Culture Collection, L strain (isolated from a patient with a chronic mastoid infection), and strain H 109 which was originally obtained from the Merck Laboratories of Rahway, New Jersey.

B. Antibiotics

1. Penicillin

The penicillin employed was crystalline penicillin G (Bristol) in vials of 100,000 unit amounts. Dilutions were made with sterile physiological saline or sterile nutrient broth. Ten ml of the diluent were added by pipette to the lyophilized penicillin yielding a final concentration of 10,000 units per ml. Dilutions were then prepared in sterile broth so that concentrations of 200, 20, 2, and 0.2 units per ml were obtained. These dilutions were employed in the various titrations to determine the end point of inhibition of the test organisms.

2. Bacitracin

The bacitracin was obtained from Dr. John

T. Goorley of Ben Venue Laboratories, Bedford, Ohio. It was supplied in vials containing 10,000 units, and dilutions were made as with penicillin to final concentrations of 200, 20, 2, and 0.2 units per ml for titrations.

C. Cultures

Stock cultures were maintained in the refrigerator on nutrient agar slants and transferred monthly. Cultures used in assays were 18 to 24 hour nutrient broth cultures grown at 37 C. Antibiotic control cultures were prepared by inoculating 10 ml of nutrient broth with 0.1 ml of a preceding 48 hour broth subculture of the original parent strain. Test cultures for developing resistance were prepared by inoculating broth with 0.5 ml of the end point tubes from the previous titration. All cultures were diluted 1 to 500 in broth for use in bacitracin and penicillin titrations. It was estimated by plate counts that from 1/2 to 1 1/2 million bacteria were added per inoculum varying with the strain. It was observed that when undiluted inocula were used, growth was obtained in higher concentrations of bacitracin than when the cultures were diluted as above.

D. Technique for Titrating the Antibiotics

The method of titrating was identical for bacitracin and penicillin, and consisted of a tube

dilution technique. Original assays were performed as follows, and all other titrations were the same except for variations in the range of the antibiotic:

(1) Concentrations of 2.0 and 0.2 units per ml were prepared.

(2) Broth tubes were set up in 2 rows of ten so that the first tube contained 1.8 ml of nutrient broth, the second tube contained 1.7 ml, the third tube contained 1.6 ml, etc., in decreasing amounts of 0.1 ml to the tenth tube, which contained 0.9 ml of the broth. The broth was distributed aseptically with a 5.0 ml pipette or from a sterile burette.

(3) To the front row the 0.2 units per ml dilution was added as follows: 0.1 ml to the first tube, 0.2 ml to the second tube, 0.3 ml to the third tube, etc., increasing by 0.1 ml amounts so that 1.0 ml was added to the tenth tube. The same technique was applied to the back row except that 2.0 units per ml was substituted for 0.2 units per ml.

(4) Then 0.1 ml of an 18 to 24 hour broth culture of the test organism was diluted in 50 ml of broth and 0.1 ml of the diluted culture was added to the tubes containing the antibiotic. This gave final concentrations of antibiotic ranging from 0.01 units per ml in hundredths to 0.1 units per ml in the front row,

and 0.1 to 1.0 units per ml in tenths in the back row. In a like manner antibiotic concentrations from 1 to 10 and 10 to 100 could be used by employing 20 units per ml and 200 units per ml respectively. Occasionally antibiotic concentrations between the usual concentrations were used, e.g., 1 to 2, 0.1 to 0.2, 2 to 3, etc.

(5) The end point tube was that tube containing the least amount of antibiotic and causing complete inhibition of the test organisms after 24 hours at 37 C. It was noted that the length of time of incubation affected the end point, as an end point at 24 hours repeatedly increased to a higher end point in 48 hours.

(6) A culture control tube containing 1.9 ml of the medium and 0.1 ml of the diluted culture accompanied the titration of each test organism.

(7) Bacitracin controls were run daily, titrating the parent strain simultaneously with the titration of each resistant organism. For example, on the day that the resistant variant of L strain and strain 209 were run, an 18 to 24 hour broth culture of the parent strains L and 209 were used in an accompanying titration. At frequent intervals penicillin controls were employed in a like manner.

E. Method for Developing Increased Resistance to the Antibiotic.

1. Preliminary Studies

Various methods were employed for increasing resistance, and one method was finally adopted as the most suitable. The method consisted of: (a) transferring 0.1 ml from the tubes containing the highest concentration of antibiotic and still yielding growth; (b) transferring 0.5 ml from the end point tubes; and (c) transferring 0.5 ml from various tubes containing higher concentrations of antibiotic than the end point tubes. These transfers were made to 10.0 ml of nutrient broth and incubated for growth or to pour agar plates from which developing colonies were picked for possible resistant variants.

2. Adopted Method

The technique finally accepted as the most suitable for this study as a result of preliminary observations was the transfer of 0.5 ml from the end point tube to 10.0 ml of nutrient broth. All tubes were incubated at 37 C for 18 to 24 hours. Growth was almost invariably obtained from end point tubes by this method. Simultaneously 0.1 ml transfers were made from 48 hour broth cultures of the original parent strains to be used an antibiotic controls. Titrations were then made using these cultures as the test organisms, and the end point tubes,this time from a higher concentration of antibiotic, were again transferred to nutrient broth as before. The assays were set up so that there were

1 to 3 tubes below and 1 to 3 tubes above the previous end point depending upon the circumstances.

F. Methods for Decreasing Resistance to the Antibiotic

Resistant cultures were treated in four ways to return them to their original susceptibility to the antibiotic. These methods consisted of maintaining resistant cultures at room temperature, in the refrigerator and in the incubator at 37 C, and of daily transfers in plain nutrient broth.

G. Cross Resistance

Cross resistance or sensitivity was determined by titrating the strain resistant to one antibiotic against the other antibiotic by the standard method used in all the other assays.

II Results

A. Original Titrations

The end points for bacitracin with the different parent strains of Staphylococcus aureus were 0.2 units per ml with the exception of the FDA strain #209 which had an initial end point at 0.09 units per ml. The initial end points with penicillin varied very slightly for the four strains, the titers falling between 0.03 and 0.06 units per ml. Table I lists the four test organisms and their initial end points. The parent

TABLE I

Initial Titrations of 4 strains of Staphylococcus aureus against bacitracin and penicillin

Organism	End Points	
	Bacitracin	Penicillin
Staphylococcus aureus 209	0.09	0.05
Staphylococcus aureus H 109	0.2	0.05
Staphylococcus aureus Oxford	0.2	0.03
Staphylococcus aureus L	0.2	0.06

strains were being transferred every 48 hours and had no exposure to the antibiotics except during the control titrations to determine the potency of the antibiotic. Despite lack of exposure to bacitracin, there were considerable variations in the end points with repeated assays of the control cultures. These variations were at first considered to be entirely due to decreases in potency of the antibiotic, thus resulting in a rise in the end point. Undoubtedly in some instances this was the explanation for rises in titer, but this explanation was insufficient to include all cases. Table II lists a series of end points of bacitracin against repeated titrations with the test organisms. At the onset of this series a new vial of bacitracin was begun. The first 10 or 11 titrations showed only slight variations, probably within the limit of technical errors. Following this group of determinations, the refrigerator, containing the entire supply of bacitracin, broke down during the weekend and it was feared that much of the potency of all the bacitracin solution had been lost. An assay on the following day, using the stored dilutions of 2 units per ml, indicated that the bacitracin had not been affected by this lack of refrigeration. The next two assays (12 and 13 for strains 209 and L; 13 and 14 for strains H 109 and (Oxford) using the same stored dilutions indicated a marked

TABLE II

End Points in One Series of Bacitracin Control Titrations

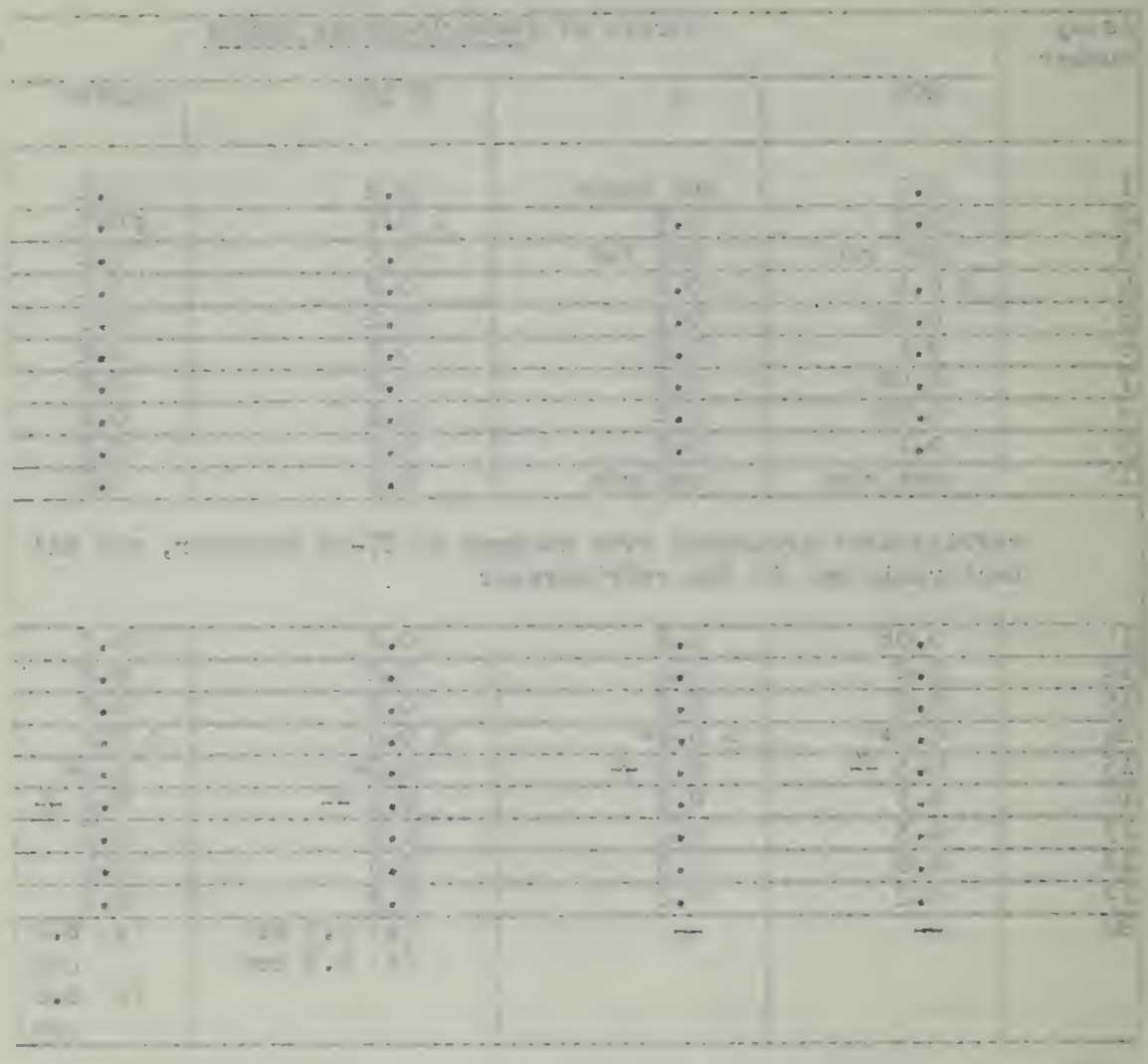
Assay Number	Strain of Staphylococcus aureus			
	209	L	H 109	Oxford
1	0.1	not begun	0.2	0.2
2	0.3	0.3	< 0.1	< 0.1
3	not run	not run	0.3	0.2
4	< 0.1	0.2	0.2	0.2
5	0.09	0.3	0.2	0.2
6	0.1	0.2	0.3	0.2
7	0.08	0.2	0.2	0.2
8	0.08	0.2	0.2	0.2
9	0.1	0.3	0.3	0.2
10	not run	not run	0.2	0.2
	refrigerator breakdown over weekend of 27-28 December, and all bacitracin was in the refrigerator			
11	0.08	0.2	0.2	< 0.1
12	0.2	0.4	0.2	0.2
13	0.2	0.5	0.3	0.2
14	0.3*	> 0.6*	> 0.4	> 0.4
15	0.3 +/-	0.5 +/-	0.5*	0.4*
16	0.3	0.3	0.5 +/-	0.4 +/-
17	0.3	0.4	0.6	0.5
18	0.2	0.3	0.5	0.4
19	0.2	0.2	0.3	0.2
20	—	—	(a) 0.3 old (b) 0.3 new	(a) 0.2 old (b) 0.2 new

The figures are end points in units per ml

* Re-diluted from stock solution containing 1000 units per ml

+/- Began use of a new vial of lyophilized bacitracin

old - Diluted to 20 units per ml prior to refrigerator stoppage
new - Prepared from most recent vial of bacitracin



loss of potency. The following titration was made after re-diluting the stock solution of 1000 units per ml up to 2 units per ml. This assay also showed a considerable loss of bacitracin activity. A fresh vial of lyophilized bacitracin was then started. The first three or four assays with the new vial surprisingly gave results comparable with the preceding titrations indicating that there was also a loss of strength in the lyophilized antibiotic. Following this the results seemed to show a return to normal for all stored bacitracin, including the most recently started vial, the previous stock solution, and the previous dilute solutions. Thus, a decrease of potency of bacitracin cannot explain the short period in which there was a rise of end point for the control cultures, since if this were the case, there could be no return toward the original lower end point. No explanation for these variations can be offered in the present study, as further investigation with this problem would be necessary for any conclusions to be reached.

Assays on penicillin gave no variations of this sort with the control cultures.

B. Rise of Resistance

1. Preliminary Series

In this series one strain of organism

(Staphylococcus aureus H 109) was carried through 20 transfers of penicillin and the resistance to penicillin developed from an original end point of 0.05 units per ml to a maximum of 10.0 units per ml which is a 200-fold increase. Table III and Graph 1.

For bacitracin two strains (h 109 and 209) run in duplicate (I and II) on alternate days were studied in this series. These were carried to 30 transfers and the resistance rose from original end points of 0.2 units per ml (one was 0.1 units per ml) to maxima of 3.0 to 7.0 units per ml, varying with the strain and particular titration. The increase in resistance ranged from 10 fold to 66,67-fold in this group with many up and down variations in all instances. The results of this study are summarized in Table III. One series (H 109 II and 209 II) of each strains in this bacitracin resistance study was carried on in the later studies. During this series it was concluded that each titration for developing resistance required an accompanying assay of the parent strain as a bacitracin control. This was not done in all cases of the preliminary series, as in some instances the control assay employed was the one performed on the previous day. The rise of resistance curves for bacitracin are shown in Figures 2 and 3,and the penicillin curve is shown in Figure 1. These results show that bacitracin fastness develops at a much slower rate than penicillin fastness, and that the bacitracin resistance rises in a straight line, although there were many variations in the end points.

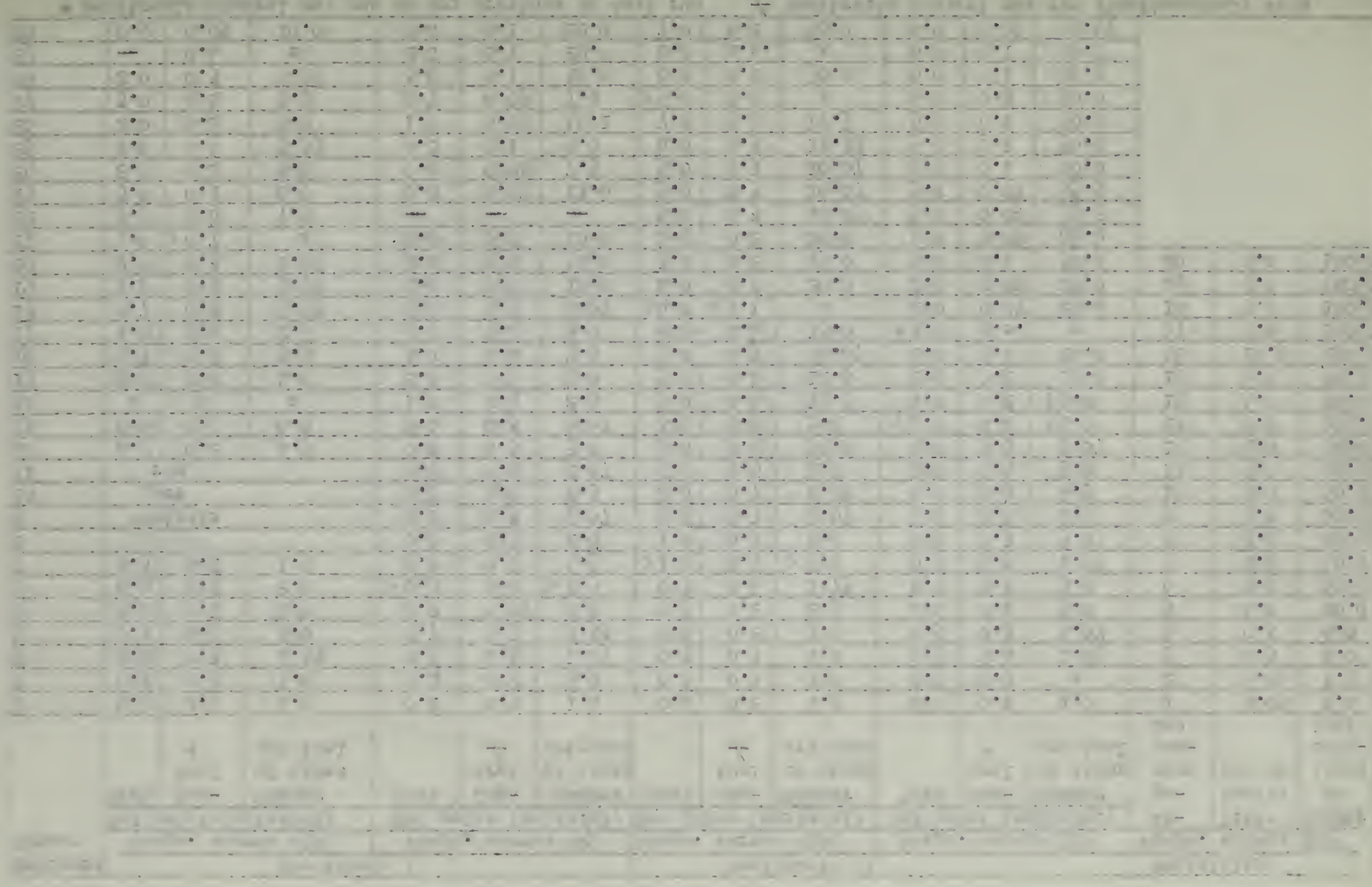
TABLE III

Rise of Resistance to Bacitracin and Penicillin

Exposure Number	Bacitracin I						Bacitracin II						Penicillin		
	Staph. aureus H109			Staph. aureus 209			Staph. aureus H109			Staph. aureus 209			Staph. aureus H109		
	End point (units/ml)			End point (units/ml)			End point (units/ml)			End point (units/ml)			Exposure number	Titer (units per ml)	Number of times original
	Test	Control *	Number of times original	Test	Control ≠	Number of times original	Test	Control ≠	Number of times original	Test	Control *	Number of times original			
1	0.2	0.2	1.0	0.2	0.2	1.0	0.2	0.2	1.0	0.1	0.1	1.0	1	0.05	1.0
2	0.2	0.2	1.0	0.4	0.2	2.0	0.1	0.1	1.0	0.2	0.3	x	2	0.06	1.2
3	0.4	0.3	1.33	0.5	0.3	1.67	0.3	0.3	1.0	0.5	0.4	1.25	3	0.3	6.0
4	0.3	0.1	3.0	0.7	0.3	2.33	0.6	0.2	3.0	0.5	0.3	1.67	4	0.3	6.0
5	0.2	0.1	2.0	0.2	0.09	2.2	0.5	0.2	2.5	0.6	0.2	3.0	5	1.0	20.0
6	0.4	0.2	2.0	> 0.5	0.2	> 2.5	0.7	0.4	1.75	0.9	0.3	3.0	6	2.0	40.0
7	0.3	0.2	1.5	0.3	0.2	1.5	> 1.0	0.5	> 2.0	0.5	0.3	1.67	7	2.0	40.0
8				0.6	0.3	2.0	1.0	0.5	2.0	0.9	0.3	3.0	8	3.0	60.0
9	controls			0.8	0.3	2.67	0.8	0.3	2.67	1.0	0.4	2.5	9	3.0	60.0
10	not			0.5	0.2	2.5	2.0	0.7	2.86	1.0	0.4	2.5	10	3.0	60.0
11	run			0.8	0.3	2.67	2.0	0.5	4.0	2.0	0.4	5.0	11	3.0	60.0
12	0.3	0.2	1.5	0.9	0.3	3.0	4.0	0.4	10.0	2.0	0.3	6.67	12	4.0	80.0
13	0.6	0.1	6.0	2.0	0.3	6.67	3.0	0.6	5.0	< 1.0	0.2	< 5.0	13	6.0	120.0
14	x	x	x	1.0	0.4	2.5	5.0	0.4	12.5	1.0	0.2	< 5.0	14	4.0	80.0
15	0.8	0.2	4.0	2.0	0.5	4.0	5.0	0.4	12.5	2.0	0.2	10.0	15	4.0	80.0
16	0.7	0.2	3.5	3.0	0.4	7.5	5.0	0.3	16.67	2.0	0.1	20.0	16	10.0	200.0
17	1.0	0.4	2.5	2.0	0.4	5.0	6.0	0.4	15.0	> 1.0	n.s.	x	17	9.0	180.0
18	0.7	0.3	2.33	2.0	0.3	6.67	< 4.0	< 0.1	x	2.0	0.07	28.6	18	9.0	180.0
19	1.0	0.3	3.33	2.0	0.2	10.0	4.0	0.2	20.0	2.0	0.08	25.0	19	9.0	180.0
20	2.0	0.3	6.67	2.0	0.2	10.0	3.0	0.2	15.0	3.0	0.1	30.0	20	8.0	160.0
21	1.0	> 0.7	x	2.0	0.2	10.0	4.0	0.2	20.0	4.0	0.09	44.4			
22	3.0	0.4	7.5	—	—	—	5.0	0.3	16.67	5.0	0.09	55.5			
23	3.0	0.5	6.0	0.8	0.07	11.4	5.0	0.3	16.67	5.0	0.09	55.5			
24	2.0	0.4	5.0	0.6	0.08	7.5	5.0	0.3	16.67	6.0	0.1	60.0			
25	2.0	0.6	3.33	0.8	0.1	8.0	4.0	0.3	13.33	5.0	0.1	50.0			
26	2.0	0.4	5.0	1.0	0.09	11.1	7.0	0.4	17.5	5.0	0.1	50.0			
27	3.0	0.4	7.5	0.7	0.09	7.8	> 5.0	0.4	x	6.0	0.3	20.0			
28	2.0	0.3	6.67	3.0	0.1	30.0	6.0	0.2	30.0	6.0	0.1	60.0			
29	—	0.4	x	2.0	0.1	20.0	6.0	n.s.	x	6.0	0.1	60.0			
30	0.9	0.09	10.0	2.0	0.1	20.0	7.0	0.2	35.0	6.0	0.09	66.67			

* Bacitracin control was run on day previous to test run ≠ Bacitracin control was run simultaneously with test run

n.s. not satisfactory



Rise of Resistance to Penicillin by Staphylococcus aureus H 109

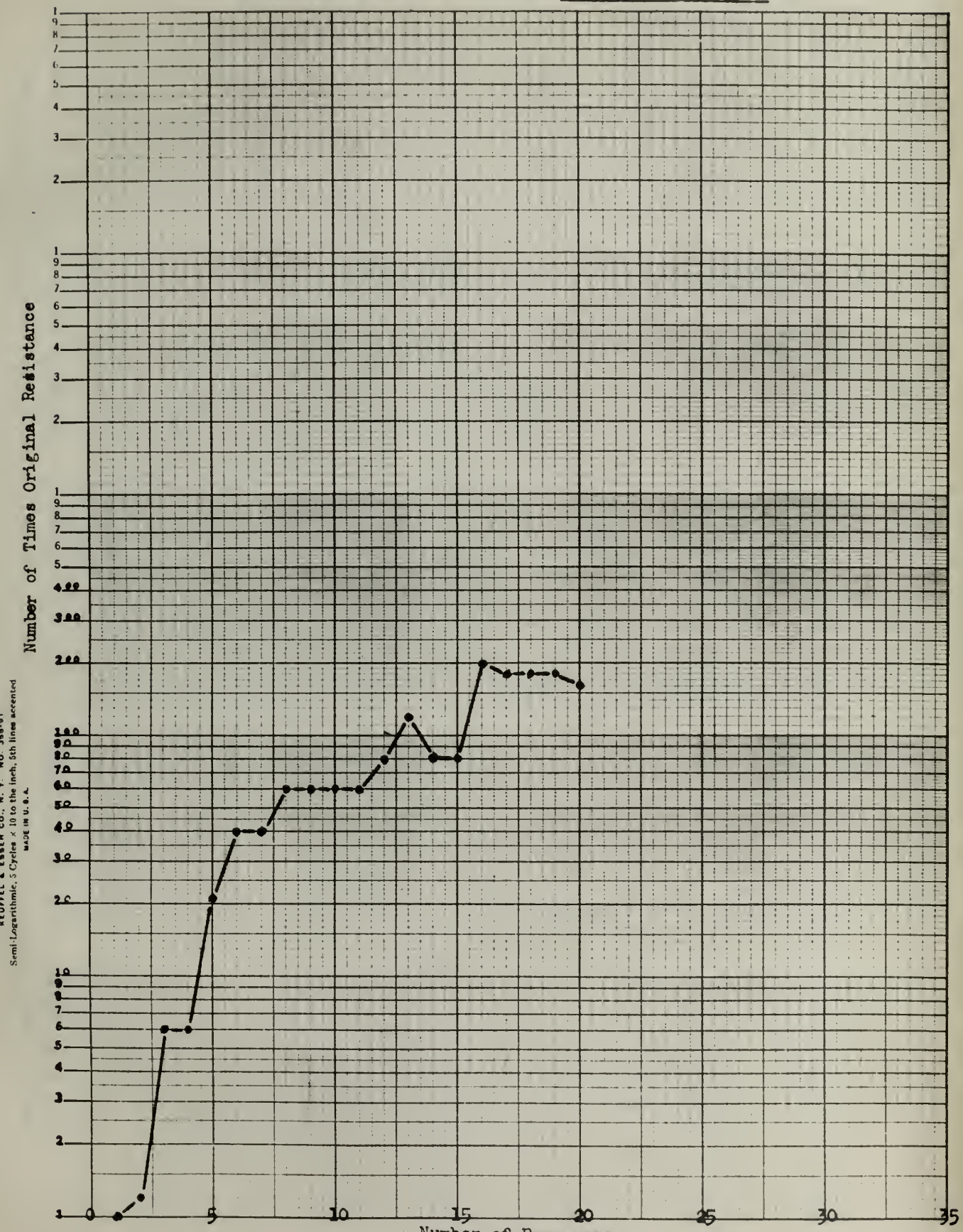


Figure 1

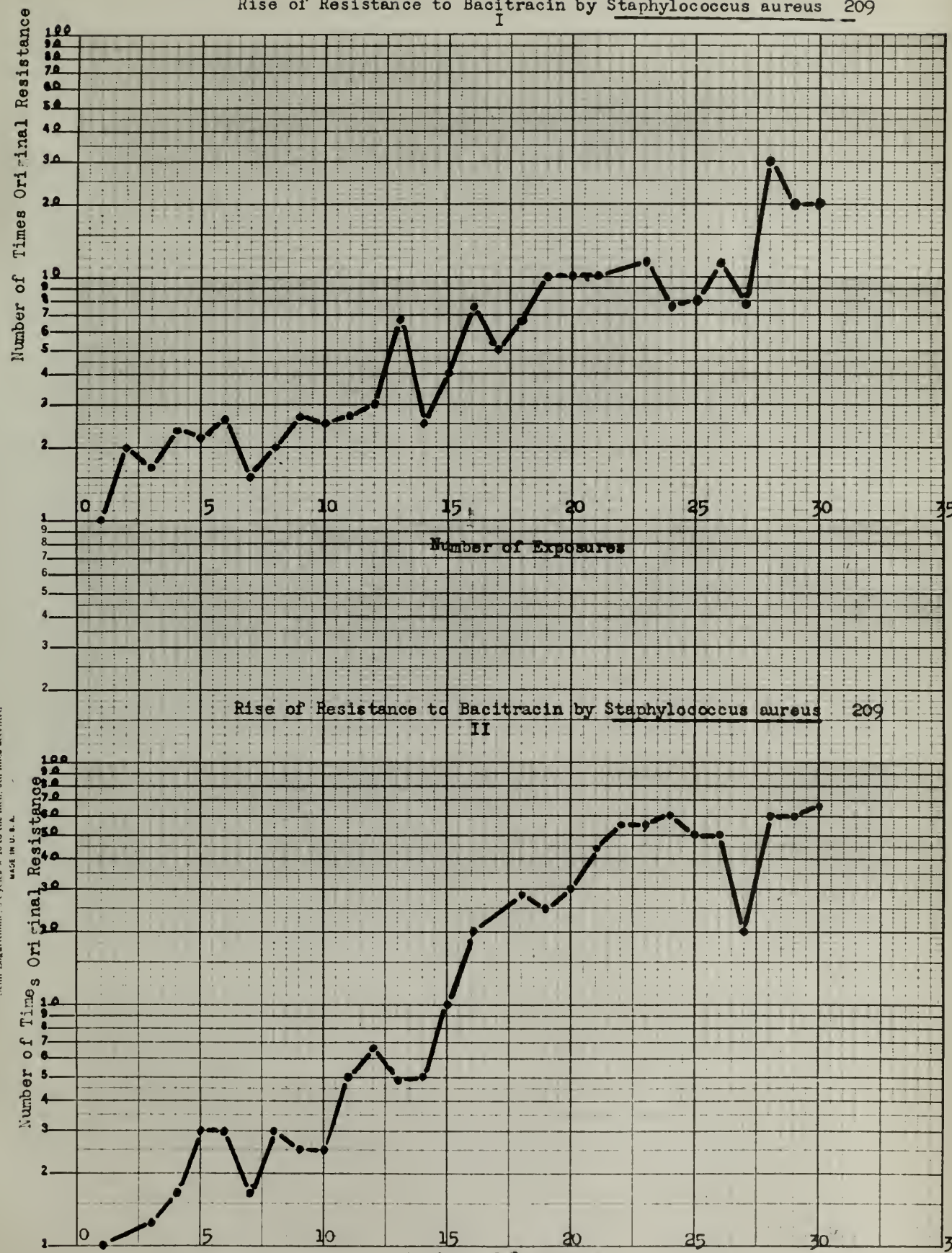

Rise of Resistance to Bacitracin by Staphylococcus aureus 209
I
Number of Times Original Resistance
Number of Exposures
Rise of Resistance to Bacitracin by Staphylococcus aureus 209
II
Number of Times Original Resistance
Number of Exposures

Figure 3

2. Later Studies

In the later studies four strains of Staphylococcus aureus (H 109, 209, Oxford, and L) were used for development of resistance to both bacitracin and penicillin. Results of these experiments are summarized in Tables IV A and B, and are shown graphically in Figures 4, 5, 6, and 7. These results confirm the results of the earlier studies, and show that the rise of resistance to penicillin is more rapid than the rise of resistance to bacitracin. The rise of resistance graphs for bacitracin were all similar with the exception of the Oxford strain, which appeared to develop very little or no resistance through 35 transfers (Figure 5). The maximum titer observed with the Oxford strain against bacitracin over the original was a 3-fold increase during the 35 exposures. Strains H 109 and 209 rose to a maximal increased resistance of 80- and 120-fold respectively with 63 exposures for the former and 62 transfers for the latter (Figure 4). The L strain of Staphylococcus aureus appears to follow the rise of resistance of strains H 109 and 209 with a 20-fold increase in 19 transfers, followed by a levelling off. There appears to be a levelling off of resistance between the 30th and 50th exposures at a resistance of approximately 50 times the original for

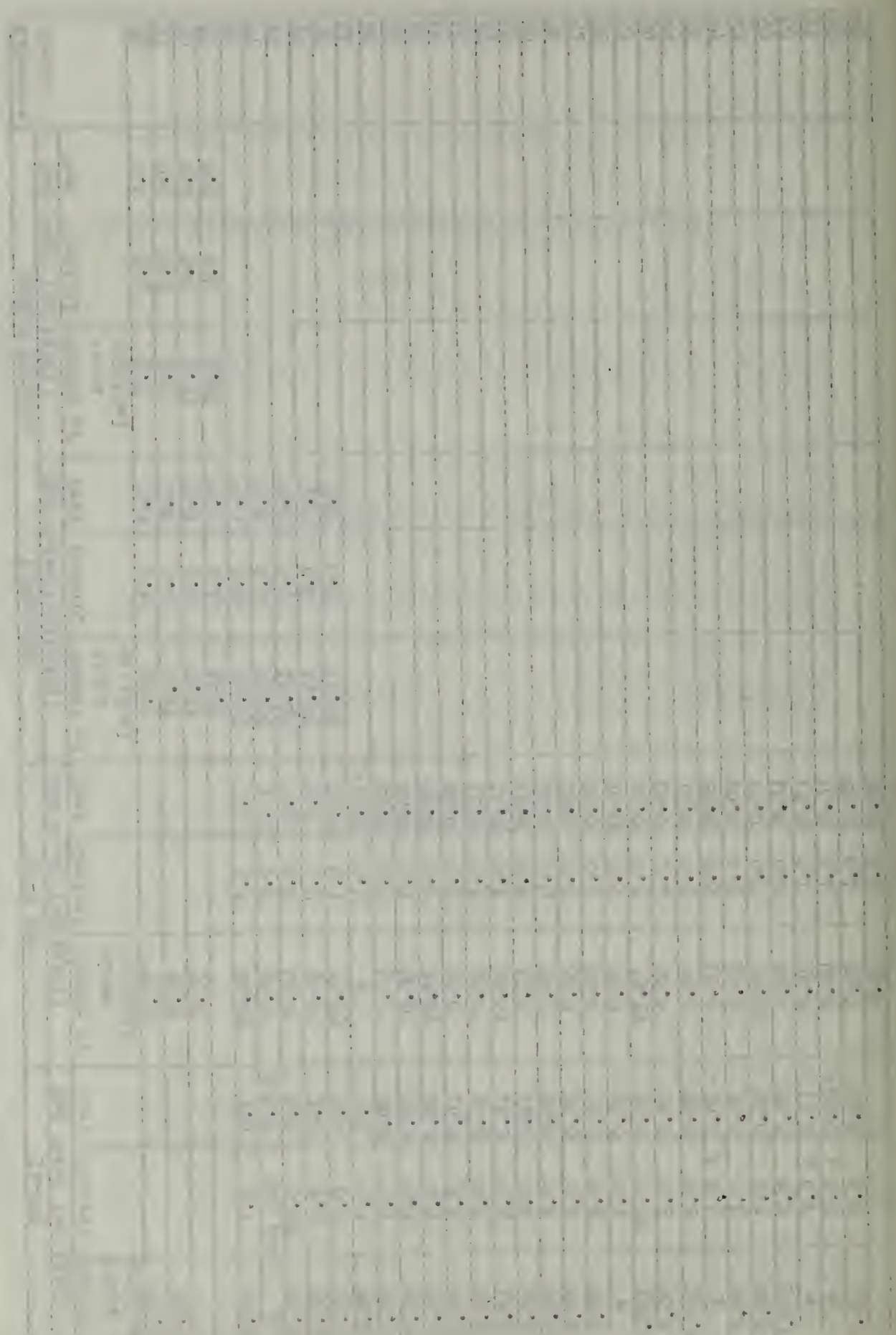
TABLE IV A

Rise of Resistance to Bacitracin by Four Strains of Staphylococcus aureus

Exposure Number	Oxford Strain			L Strain			II-H109			II-209		
	End Point (units/ml)			End Point (units/ml)			End Point (units/ml)			End Point (units/ml)		
	Test	Control	Number of times original	Test	Control	Number of times original	Test	Control	Number of times original	Test	Control	Number of times original
1	0.2	0.2	1.0	0.3	0.3	1.0	0.2	0.2	1.0	0.1	0.1	1.0
2	0.3	0.2	1.5	0.3	0.2	1.5	0.1	0.1	1.0	0.2	0.3	x
3	0.3	0.2	1.5	>0.5	0.3	> 1.67	0.3	0.3	1.0	0.5	0.4	1.25
4	0.5	0.2	2.5	0.9	0.2	4.5	0.6	0.2	3.0	0.5	0.3	1.67
5	0.5	0.2	2.5	0.9	0.2	4.5	0.5	0.2	2.5	0.6	0.2	3.0
6	0.4	0.2	2.0	0.8	< 0.1	> 8.0	0.7	0.4	1.75	0.9	0.3	3.0
7	0.5	0.2	2.5	>1.0	0.2	> 5.0	1.0	0.5	2.0	0.5	0.3	1.67
8	0.6	0.2	3.0	2.0	0.3	6.67	1.0	0.5	2.0	0.9	0.3	3.0
9	x	x	x	x	x	x	0.8	0.3	2.67	1.0	0.4	2.5
10	>0.6	0.2	> 3.0	1.0	0.2	5.0	2.0	0.7	2.86	1.0	0.4	2.5
11	0.4	> 0.4	x	3.0	0.4	7.5	2.0	0.5	4.0	2.0	0.4	5.0
12	0.5	0.4	1.2	4.0	0.5	8.0	4.0	0.4	10.0	2.0	0.3	6.67
13	0.7	0.4	1.75	5.0	>0.6	< 8.33	3.0	0.6	5.0	1.0	0.2	5.0
14	0.7	0.5	1.4	4.0	0.5	8.0	5.0	0.4	12.5	1.0	0.2	5.0
15	0.7	0.4	1.75	4.0	0.3	13.33	5.0	0.4	12.5	2.0	0.2	10.0
16	0.5	0.2	2.5	5.0	0.5	10.0	5.0	0.3	16.67	2.0	0.1	20.0
17	0.4	0.2	2.0	4.0	0.4	10.0	6.0	0.4	15.0	>1.0	n.s.	x
18	>0.6	0.4	> 1.5	3.0	0.3	10.0	< 4.0	< 0.1	x	2.0	0.07	28.6
19	<0.4	0.2	< 2.0	4.0	0.2	20.0	4.0	0.2	20.0	2.0	0.08	25.0
20	0.4	0.3	1.33	6.0	0.4	15.0	3.0	0.2	15.0	3.0	0.1	30.0
21	0.8	0.3	2.67	7.0	0.5	14.0	4.0	0.2	20.0	4.0	0.09	44.4
22	0.7	0.3	2.33	<5.0	0.4	< 12.5	5.0	0.3	16.67	5.0	0.09	55.5
23	0.6	0.2	3.0	5.0	0.6	8.33	5.0	0.3	16.67	5.0	0.09	55.5
24	0.6	0.2	3.0	5.0	0.5	10.0	5.0	0.3	16.67	6.0	0.1	60.0
25	0.4	0.3	1.33	3.0	0.2	15.0	4.0	0.3	13.33	5.0	0.1	50.0
26	0.7	0.4	1.75	2.0	0.2	10.0	7.0	0.4	17.5	5.0	0.1	50.0
27	0.6	0.5	1.2	4.0	0.3	13.33	< 5.0	0.4	x	6.0	0.3	20.0
28	0.6	0.3	2.0	5.0	0.4	12.5	6.0	0.2	30.0	6.0	0.1	60.0
29	0.6	0.4	1.5	7.0	0.6	11.57	6.0	n.s.	x	6.0	0.1	60.0
30	0.3	0.2	1.5	6.0	0.7	8.57	7.0	0.2	35.0	6.0	0.09	66.67
31	0.6	0.3	2.0	6.0	0.7	8.57			50.0			66.67

TABLE IV A Continued

Exposure Number	Oxford Strains			L Strains			II-H 109			II-209		
	End Point (units/ml)			End Point (units/ml)			End Point (units/ml)			End Point (units/ml)		
	Test	Control	Number of times original	Test	Control	Number of times original	Test	Control	Number of times original	Test	Control	Number of times original
32	0.6	0.4	1.5	4.0	0.4	10.0			55.0			60.0
33	0.6	0.3	2.0	4.0	0.6	6.67			50.0			62.5
34	0.8	0.6	1.33	5.0	0.6	8.33			55.0			
35	0.9	0.4	2.25	5.0	0.4	12.5						
36				5.0	0.3	16.67	8.0	0.3	26.67	7.0	0.1	70.0
37				8.0	0.6	13.33	10.0	0.2	50.0	< 4.0	not run	x
38				8.0	0.7	11.43	8.0	0.2	40.0	4.0	0.08	50.0
39				7.0	0.6	11.67	6.0	0.2	30.0	7.0	0.2	35.0
40				8.0	0.7	11.43	10.0	0.3	33.33	7.0	0.2	35.0
41							> 10.0	> 0.4	x	9.0	0.3	30.0
42							20.0	0.5	40.0	20.0	0.3	66.67
43							20.0	0.5	40.0	20.0	0.3	66.67
44							20.0	0.6	33.67	20.0	0.3	66.67
45							20.0	0.5	40.0	14.0	0.2	70.0
46							12.0	0.3	40.0	16.0	0.2	80.0
47							14.0	0.3	46.67	16.0	0.4	40.0
48							16.0	0.5	32.0	18.0	0.4	45.0
49							20.0	0.4	50.0	20.0	0.3	66.67
50							20.0	0.4	50.0	18.0	0.2	90.0
51							20.0	0.5	40.0	18.0	0.3	60.0
52							10.0	0.5	20.0	< 16.0	< 0.1	x
53							10.0	0.3	33.33	10.0	0.08	125.0
54							20.0	0.4	50.0	18.0	0.2	90.0
55							18.0	0.3	60.0	20.0	0.2	100.0
56							20.0	0.5	40.0	> 20.0	> 0.5	x
57							20.0	0.5	40.0	30.0	0.4	75.0
58							16.0	0.6	26.67	24.0	0.3	80.0
59							16.0	0.2	80.0	20.0	0.2	100.0
60							18.0	0.4	45.0	> 18.0	> 0.2	x
61							16.0	0.4	40.0	> 20.0	> 0.2	x
62							20.0	0.4	50.0	24.0	0.2	120.0
63							20.0	0.5	40.0			



Rise of Resistance to Bacitracin by Staphylococcus aureus H109 (II)

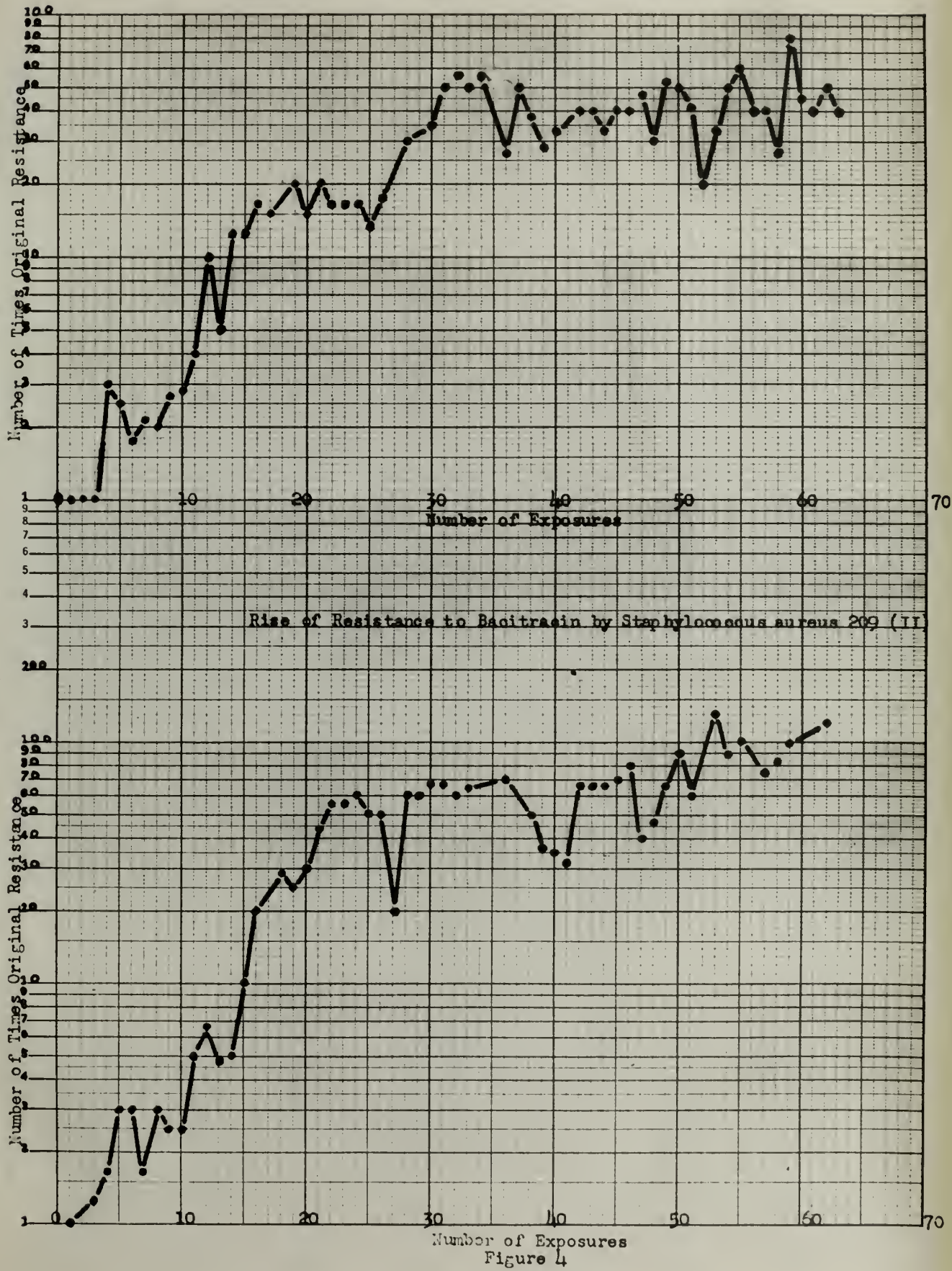
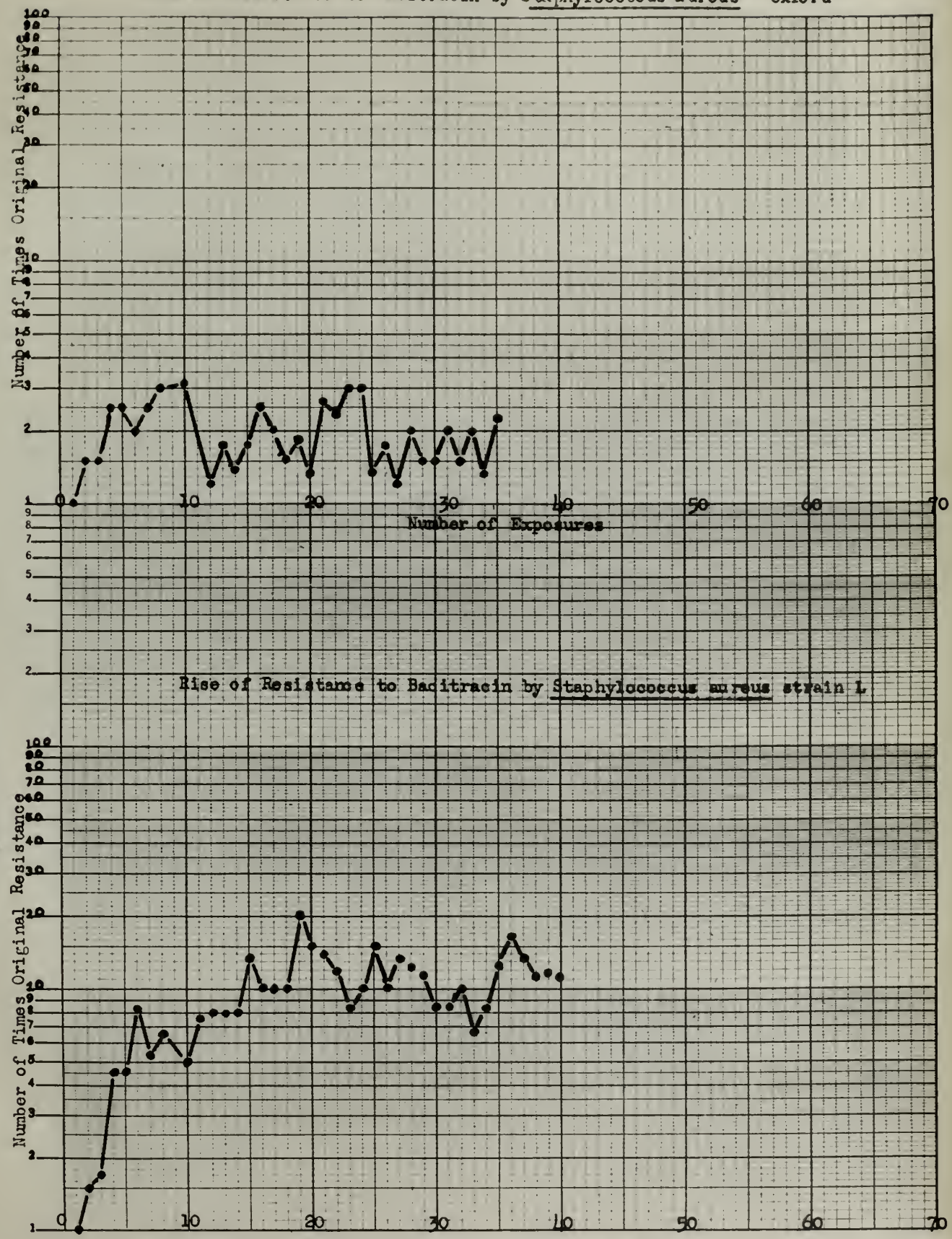


Figure 4



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Semi-Logarithmic, 5 Cycles × 10 to the inch, 5th lines accented
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Figure 5

Rise in Bacitracin Resistance in Percentage of Peak against Number of Exposures

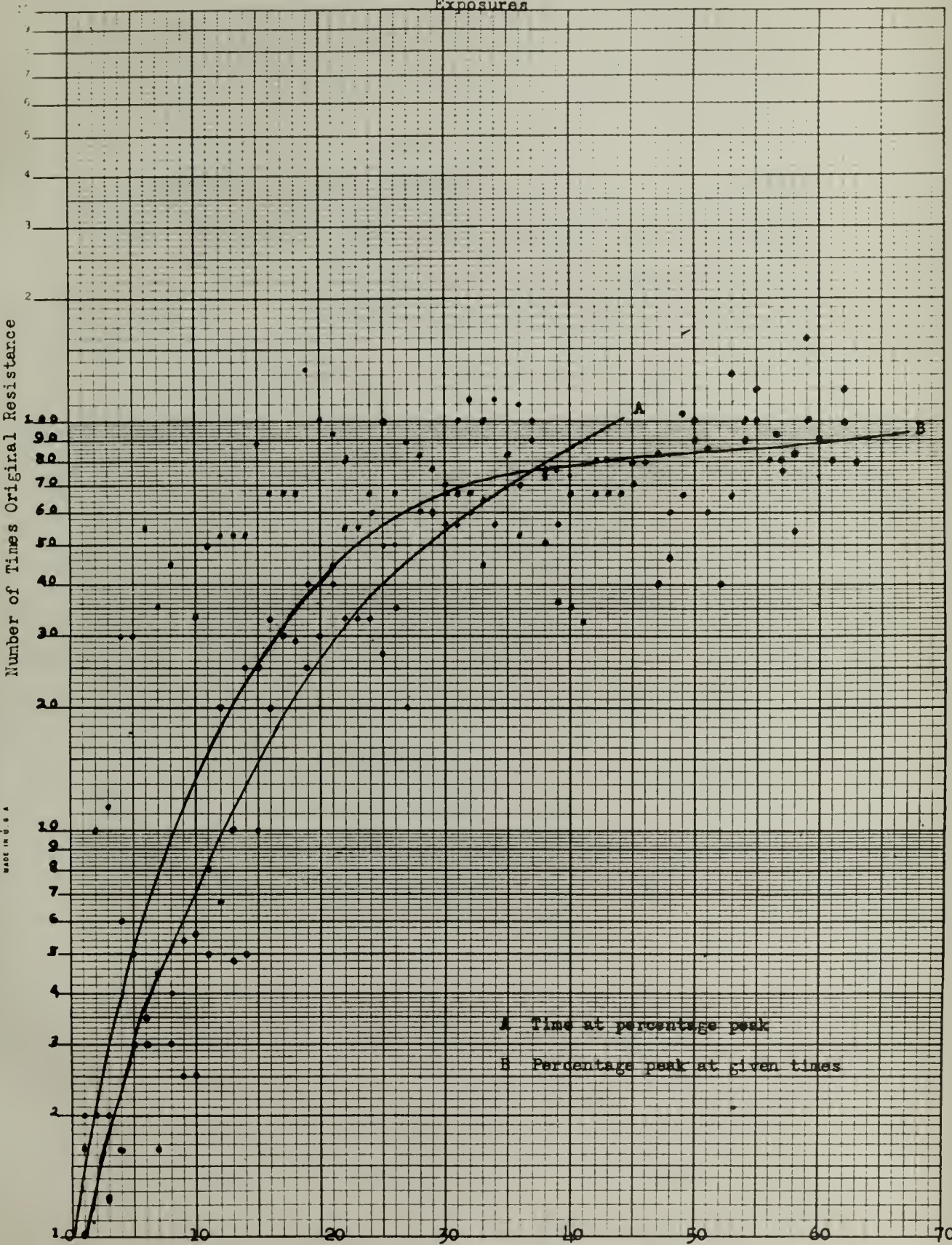


Figure 5 A

strain H 109 and at about the 60-fold resistance level for strain 209, although there appears to be a secondary rise with strain 209 after the 50th exposure. Figure 5 A is a composite of three strains (L, H 109 and 209) of Staphylococcus aureus in percentage of peak resistance to bacitracin. The Oxford strain did not develop any appreciable resistance and was, therefore, not included in the composite.

The rise of resistance to penicillin appears to be slightly more rapid and to a somewhat greater extent than bacitracin. The maximal titer obtained for any one of the strains using bacitracin was 120 times the original titer, and this occurred after 62 transfers, and for one strain only (strain 209); whereas, the maximal titer obtained for penicillin was 400 times the original titer and this occurred after only 32 transfers, also with strain 209. In addition, all strains rose to at least 100 times the original resistance to penicillin, and not more than 31 transfers were required in any instance, while only one strain, as mentioned above, reached 100-fold resistance to bacitracin. The rise in resistance to penicillin is shown in tabular form in Table IV B and graphically in Figures 6 and 7. All four strains appeared to attain their maximal resistance to penicillin at approximately 30 exposures, and this was followed by a slight decrease for a few transfers. This drop was for

TABLE IV B

Rise of Resistance to Penicillin by Four Strains of Staphylococcus aureus

Exposure Number	Strain 209		Strain H 109		Oxford Strain		L Strain	
	End Point (units/ml)	Number of times original resistance	End Point (units/ml)	Number of times original resistance	End Point (units/ml)	Number of times original resistance	End Point (units/ml)	Number of times original resistance
1	0.05	1.0	0.05	1.0	0.03	1.0	0.06	1.0
2	0.06	1.2	0.07	1.4	0.05	1.67	0.05	x
3	0.1	2.0	0.09	1.8	0.09	3.0	0.08	1.33
4	0.2	4.0	0.1	2.0	0.1	3.33	0.1	1.67
5	0.3	6.0	0.2	4.0	0.2	6.67	0.2	3.33
6	0.3	6.0	0.1	2.0	0.1	3.33	0.2	3.33
7	0.3	6.0	0.2	4.0	0.09	3.0	0.2	3.33
8	0.3	6.0	0.4	8.0	0.2	6.67	0.2	3.33
9	0.3	6.0	0.4	8.0	0.2	6.67	0.2	3.33
10	0.3	6.0	0.4	8.0	0.2	6.67	0.3	5.0
11	0.5	10.0	0.5	10.0	0.3	10.0	< 0.1	x
12	0.5	10.0	0.7	14.0	0.3	10.0	0.3	5.0
13	0.4	8.0	0.8	16.0	0.4	13.33	0.4	6.67
14	0.6	12.0	0.9	18.0	0.5	16.67	x	x
15	x	x	1.0	20.0	0.5	16.67	0.5	8.33
16	0.8	16.0	2.0	40.0	0.5	16.67	0.5	8.33
17	0.8	16.0	2.0	40.0	0.5	16.67	0.5	8.33
18	1.0	20.0	2.0	40.0	0.6	20.0	0.5	8.33
19	1.0	20.0	2.0	40.0	0.6	20.0	0.5	8.33
20	2.0	40.0	2.0	40.0	0.6	20.0	0.5	8.33
21	3.0	60.0	2.0	40.0	0.6	20.0	0.7	11.67
22	3.0	60.0	2.0	40.0	0.8	26.67	0.9	15.0
23	4.0	80.0	2.0	40.0	0.7	23.33	2.0	33.33
24	4.0	80.0	2.0	40.0	0.9	30.0	2.0	33.33
25	6.0	120.0	3.0	60.0	1.0	33.33	3.0	50.0
26	5.0	100.0	3.0	60.0	1.0	33.33	3.0	50.0
27	7.0	140.0	3.0	60.0	1.0	33.33	3.0	50.0
28	7.0	140.0	2.0	40.0	2.0	66.67	4.0	66.67
29	7.0	140.0	5.0	100.0	2.0	66.67	7.0	116.67
30	10.0	200.0	4.0	80.0	—	—	10.0	166.67
31	10.0	200.0	3.0	60.0	3.0	100.0	10.0	166.67
32	20.0	400.0	1.0	20.0	3.0	100.0	10.0	166.67
33	8.0	160.0	2.0	40.0	3.0	150.0	7.0	116.67
34	10.0	200.0	2.0	40.0	2.0	66.67	4.0	66.67

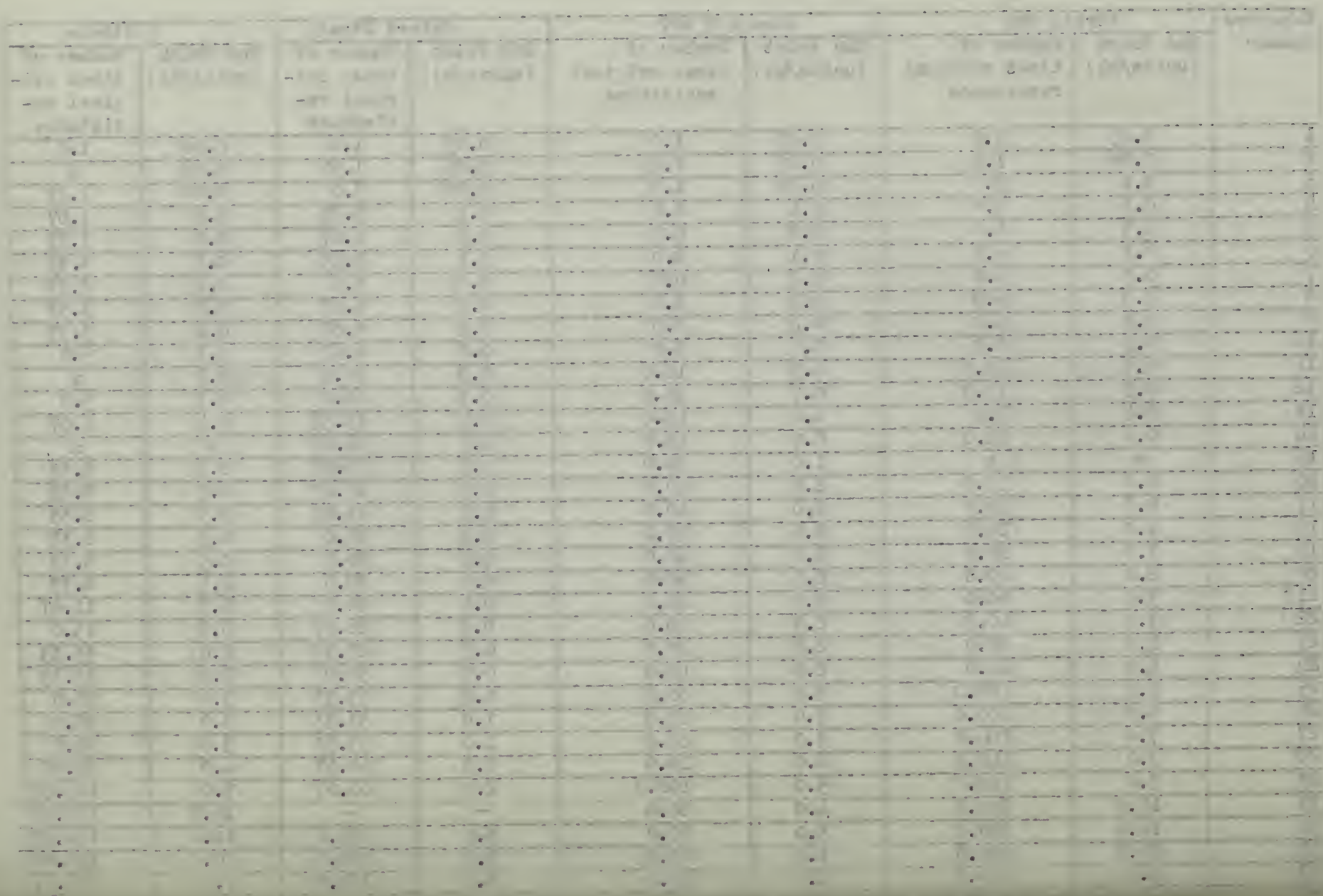
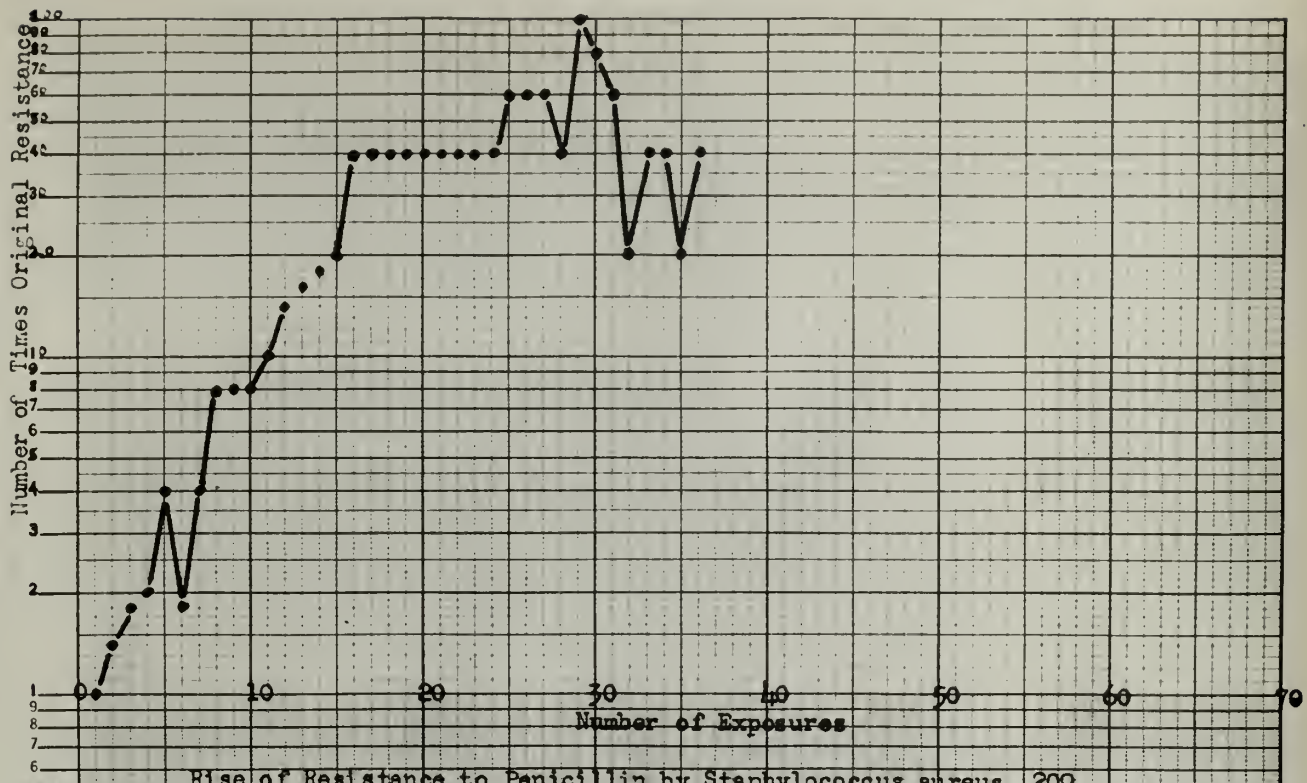


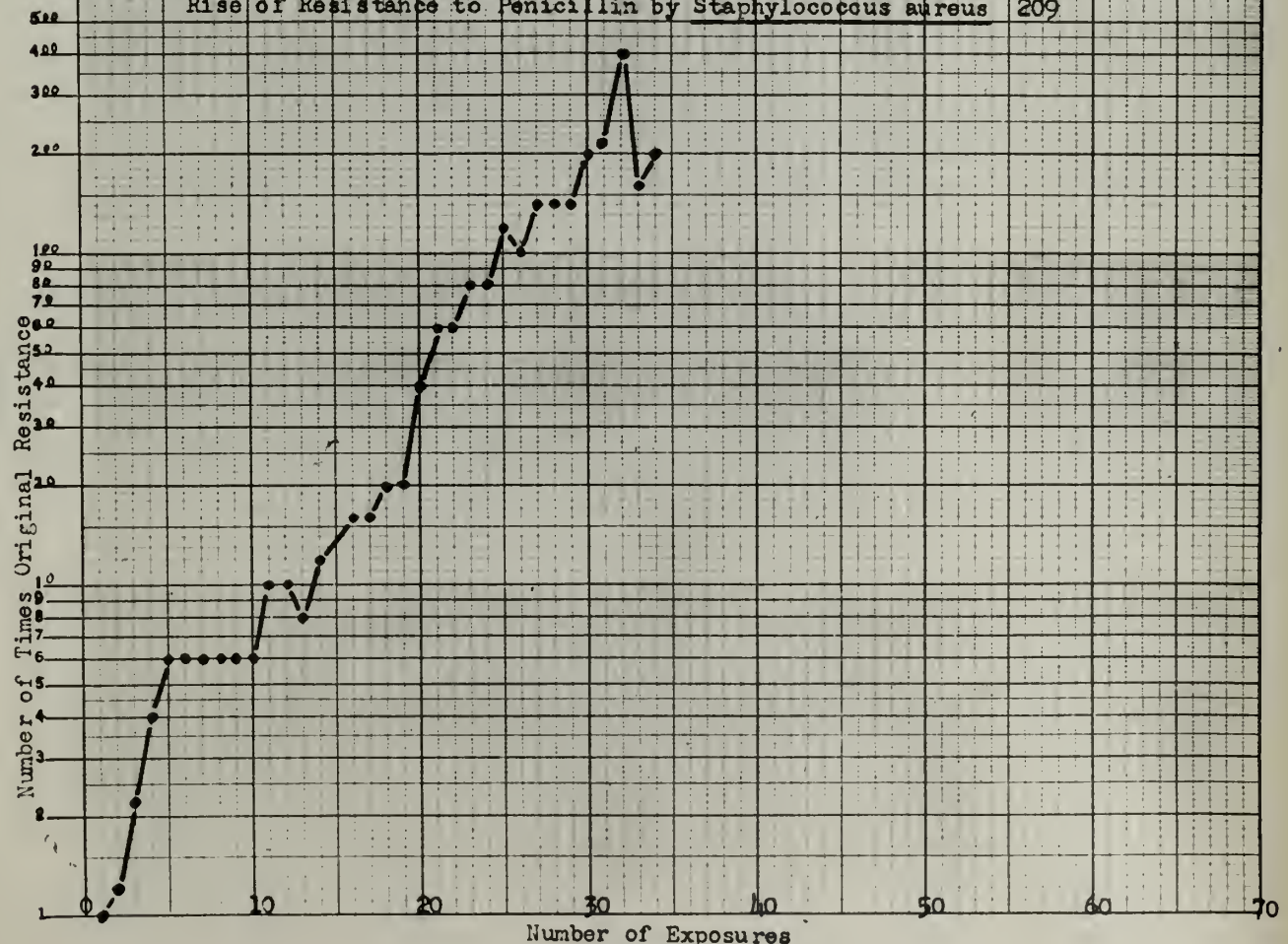
TABLE IV B continued

Exposure Number	Strain 209		Strain H 109		Oxford Strain		L Strain	
	End Point (units/ml)	Number of times original resistance	End Point (units/ml)	Number of times original resistance	End Point (units/ml)	Number of times original resistance	End Point (units/ml)	Number of times original resistance
35			1.0	20.0	1.0	33.33	5.0	83.33
36			2.0	40.0	2.0	66.67	5.0	83.33
37					1.0	33.33	8.0	133.33
38					2.0	66.67	9.0	150.0

Rise of Resistance to Penicillin by Staphylococcus aureus H 109



Rise of Resistance to Penicillin by Staphylococcus aureus 209



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Semi-Logarithmic, 3 Cycles x 10 to the inch, 5th lines accented
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Figure 6

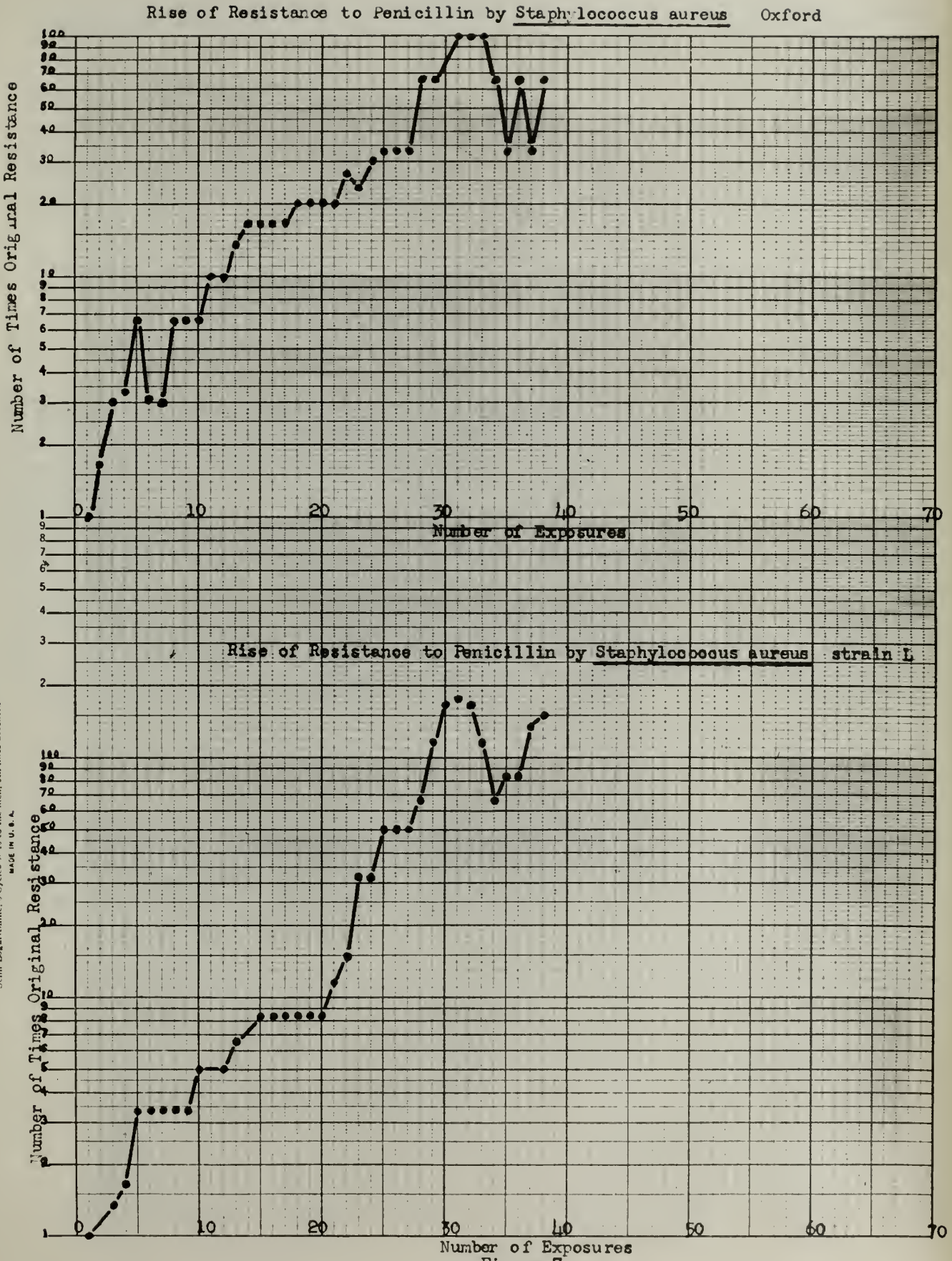

Rise of Resistance to Penicillin by Staphylococcus aureus Oxford
Number of Times Original Resistance
Number of Exposures
Rise of Resistance to Penicillin by Staphylococcus aureus strain L
Number of Times Original Resistance
Number of Exposures

Figure 7

Rise in Penicillin Resistance in Percentage of Peak against Number of Exposures

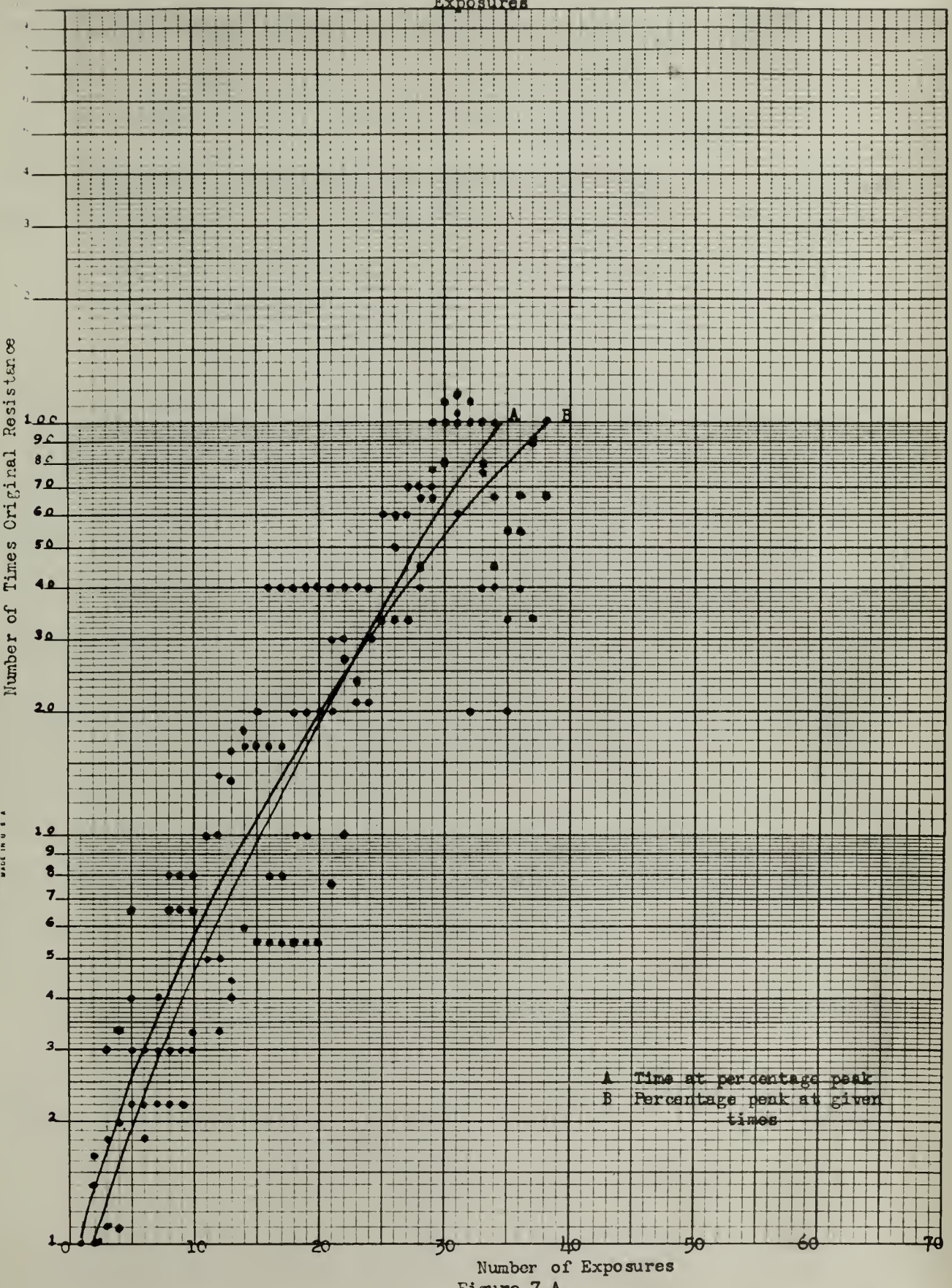


Figure 7 A

a short time only, since a second rise seemed to be commencing just prior to the discortinuation of the rise in resistance studies. Figure 7 A is a composite of the four strains of Staphylococcus aureus in percentage of peak resistance to penicillin.

C. Drop of Resistance

1. Preliminary Series

Penicillin only was studied in the preliminary series. The drop in resistance to penicillin by Staphylococcus aureus H 109 was rapid by all methods employed. It was slightly more rapid by daily transfers of the penicillin resistant strains through plain nutrient broth than by maintaining the culture at either incubator, refrigerator, or room temperature, and was slightly slower by maintenance at room temperature than by the other methods. The culture maintained in the refrigerator dropped at a more rapid rate than the one maintained at room temperature, but was slower than the other two. The results are summarized in Table V and illustrated in Figure 8. It can be seen that, from a titer of 160.0 times the natural end point with that organism, there was a drop towards normal so that the titer was nearly at its original level in from 14 to 39 days varying with the type of treatment of the resistant culture.

TABLE V

Drop in Penicillin Resistance of Staphylococcus aureus H 109

Number of days	Drop in Resistance							
	Daily Transfer		Maintained at 37 C		Maintained in Refrigerator		Maintained at Room Temperature	
	Titer (units/ml)	Number of times original	Titer (units/ml)	Number of times original	Titer (units/ml)	Number of times original	Titer (units/ml)	Number of times original
0	8.0	160.0	8.0	160.0	8.0	160.0	8.0	160.0
7	1.0	20.0	0.1	2.0	10.0	200.0	9.0	180.0
9	1.0	20.0	0.5	10.0	not run	not run	not run	not run
14	0.3	6.0	0.7	14.0	not run	not run	not run	not run
18	not run	not run	not run	not run	4.0	80.0	1.0	20.0
21	0.3	6.0	0.3	6.0	1.0	20.0	5.0	100.0
28	0.2	4.0	0.4	8.0	0.5	10.0	3.0	60.0
29	not run	not run	0.2	4.0	not run	not run	not run	not run
32	not run	not run	not run	not run	0.5	10.0	4.0	80.0
33	0.2	4.0	0.2	4.0	0.3	6.0	1.0	20.0
39	0.3	6.0	0.4	8.0	0.08	1.6	0.6	12.0

Rise and Fall in Resistance to Penicillin by Staphylococcus aureus H 109

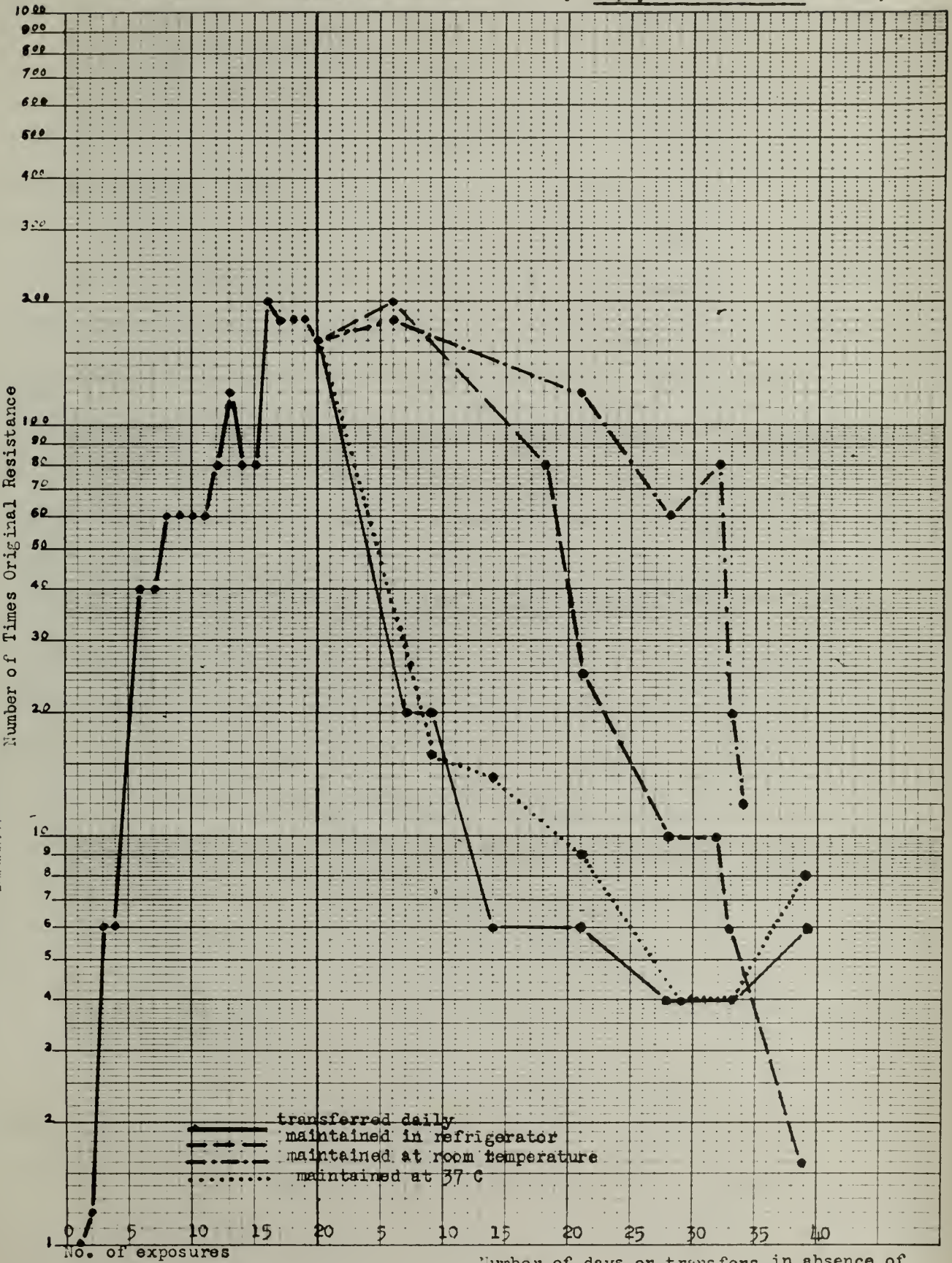


Figure 8

2. Later Studies

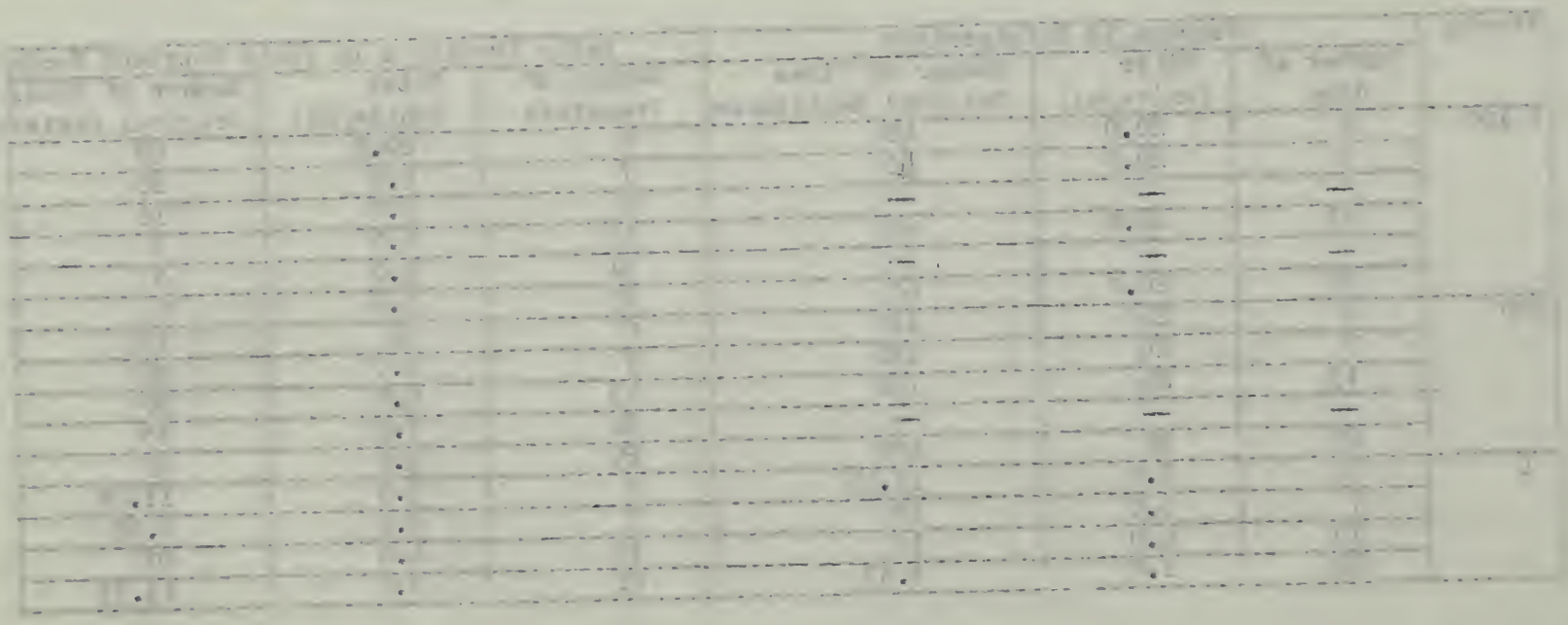
Drop in resistance studies with bacitracin indicate that there is a fairly rapid drop toward normal if the cultures are being transferred daily in plain nutrient broth in the absence of bacitracin. The results are summarized in Table VI and shown graphically in Figures 9 A, 9 B, and 9 C. It can be seen that for strain H 109 there is a rapid decrease in resistance from 40 times to 10 times the original in 7 daily transfers, followed by slower drops to 8 and 6 times after 14 and 21 transfers respectively. Strain 209 dropped very rapidly from 120 times its original resistance to 30 times the original resistance in 7 transfers, and it finally reached 20 times its original resistance after 29 transfers in plain nutrient broth. Strain L gave a different picture; its drop was only slight at the beginning, dropping from about 11 to about 8.5 times the original resistance in 7 days, showing a return to 10 after 14 transfers so that the decrease in resistance remained only very slight. In 21 days there appeared to be a slight rise to 13.3 times the original resistance.

Looking at the same table and graphs, we find that there was little or no drop in resistance following storage in the refrigerator of all three strains.

TABLE VI

Decrease in Resistance to Bacitracin by 3 Strains of Staphylococcus aureus

Strain	Storage in Refrigerator			Daily Transfers in Plain Nutrient Broth		
	Number of days	Titer (units/ml)	Number of Times Original Resistance	Number of Transfers	Titer (units/ml)	Number of Times Original Resistance
H 109	0	20.0	40	0	20.0	40
	7	20.0	40	7	5.0	10
	—	—	—	8	5.0	10
	13	20.0	40	13	4.0	8
	—	—	—	21	3.0	6
	29	20.0	40	29	3.0	6
209	0	24	120	0	24	120
	7	24	120	7	6.0	30
	13	24	120	13	6.0	30
	—	—	—	21	7.0	35
	29	20	100	29	4.0	20
L	0	8.0	11.43	0	8.0	11.43
	7	6.0	10	7	5.0	8.33
	14	4.0	10	14	4.0	10
	21	1.0	3.33	21	3.0	13.33



Rise and Fall in Resistance to Bacitracin by Staphylococcus aureus

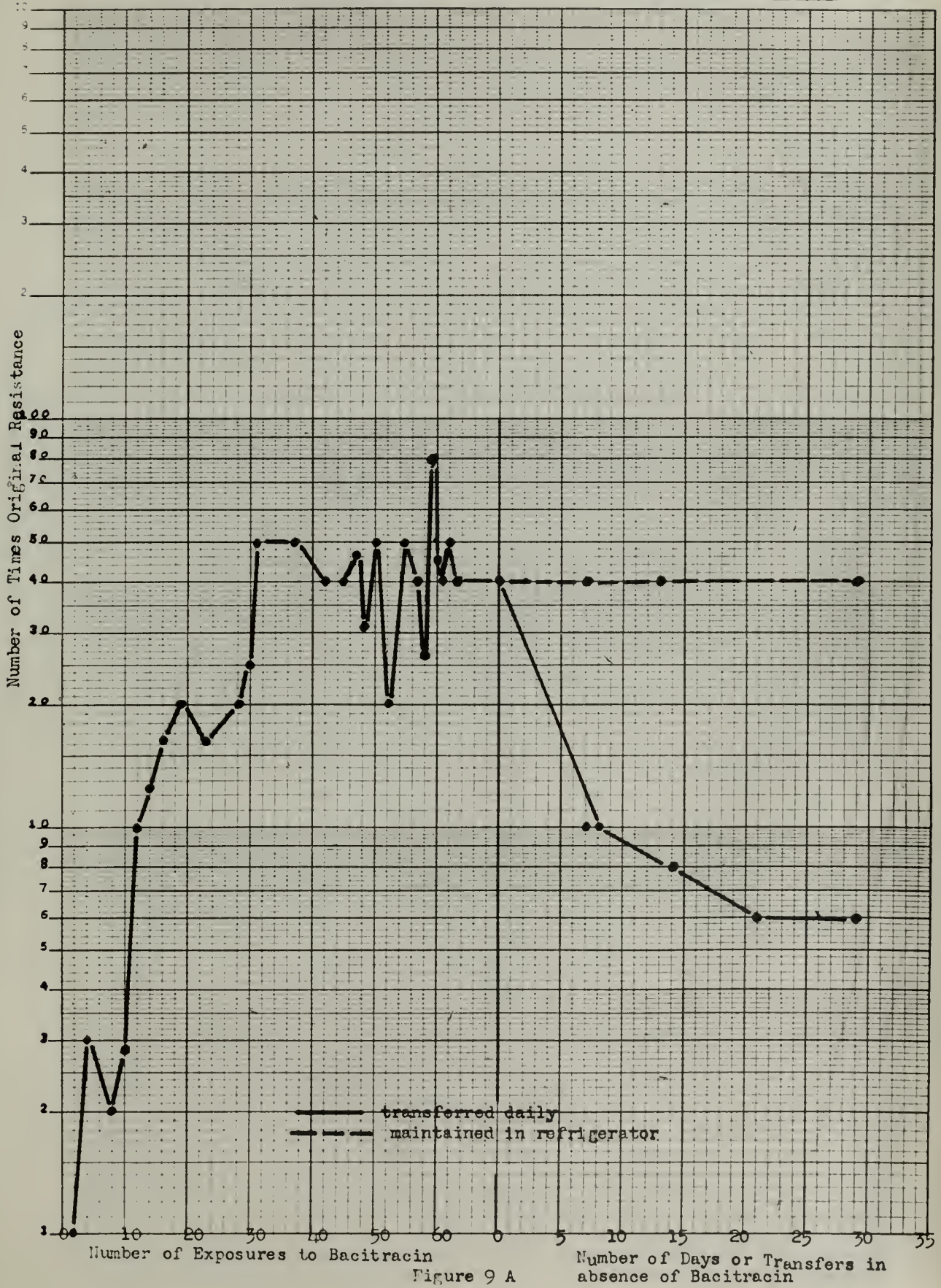


Figure 9 A

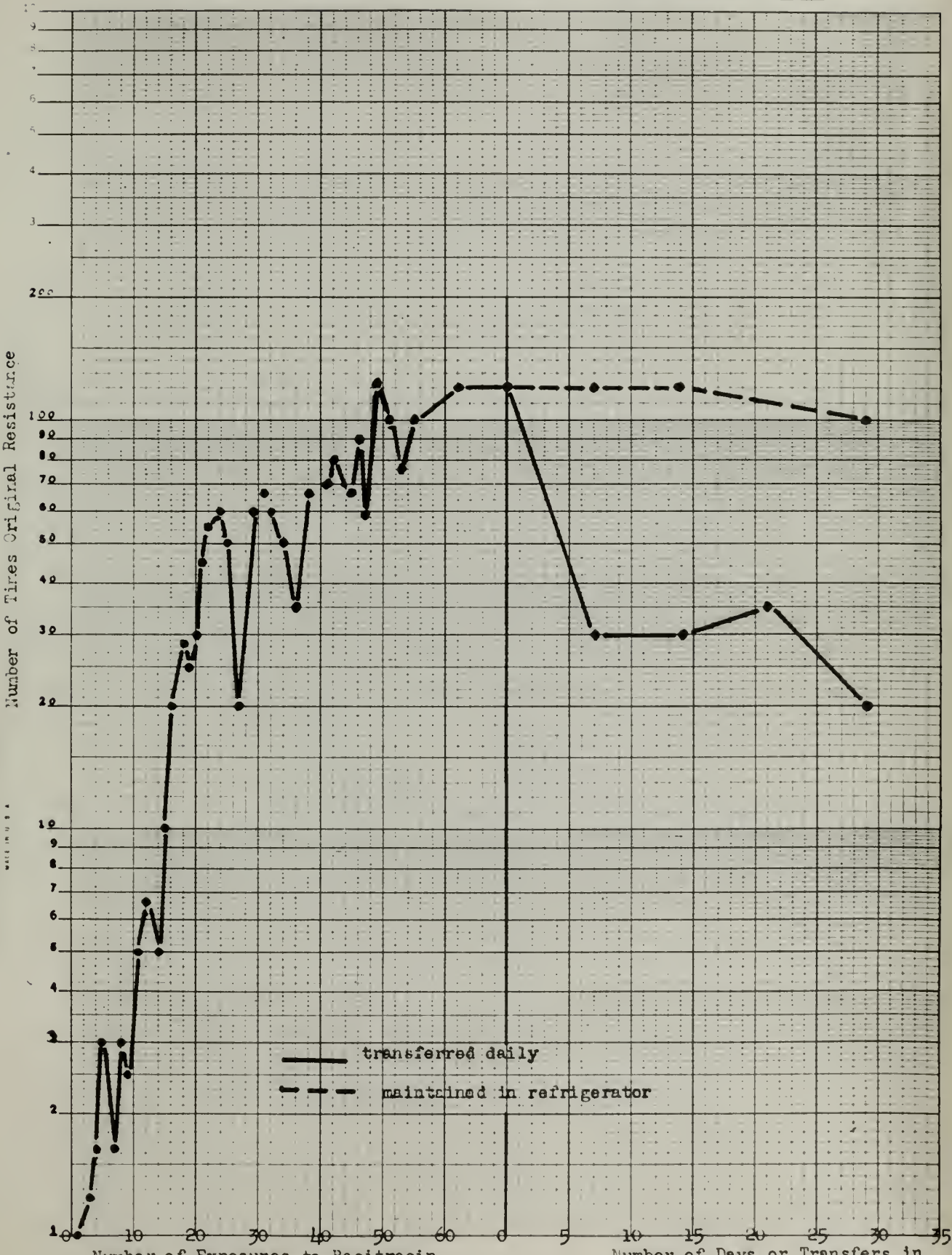

Number of Times Original Resistance
transferred daily
maintained in refrigerator
Number of Exposures to Bacitracin
Number of Days or Transfers in absence of Bacitracin

Figure 9 B

Rise and Fall in Resistance to Bacitracin by Staphylococcus aureus L strain

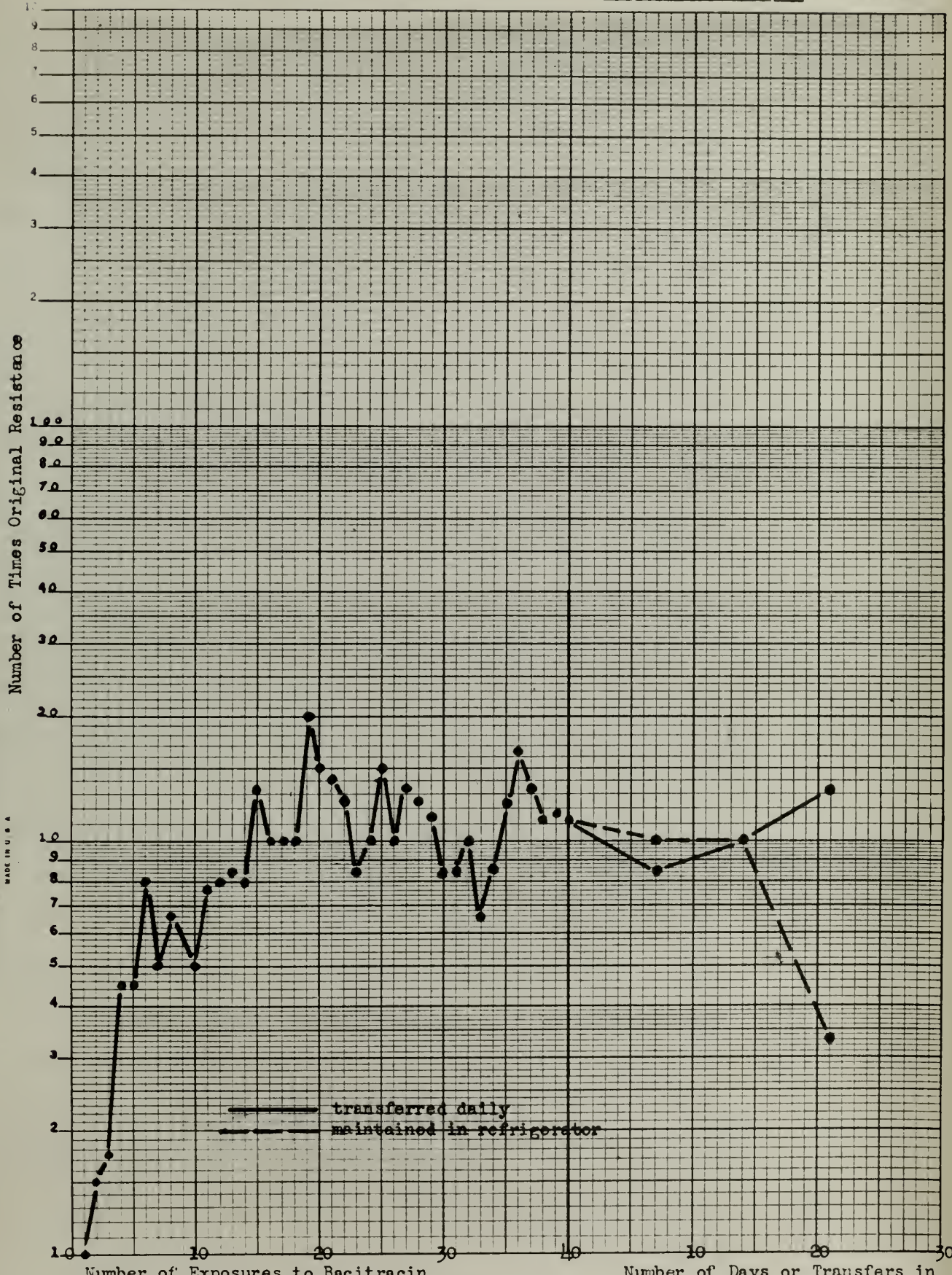


Figure 9 C

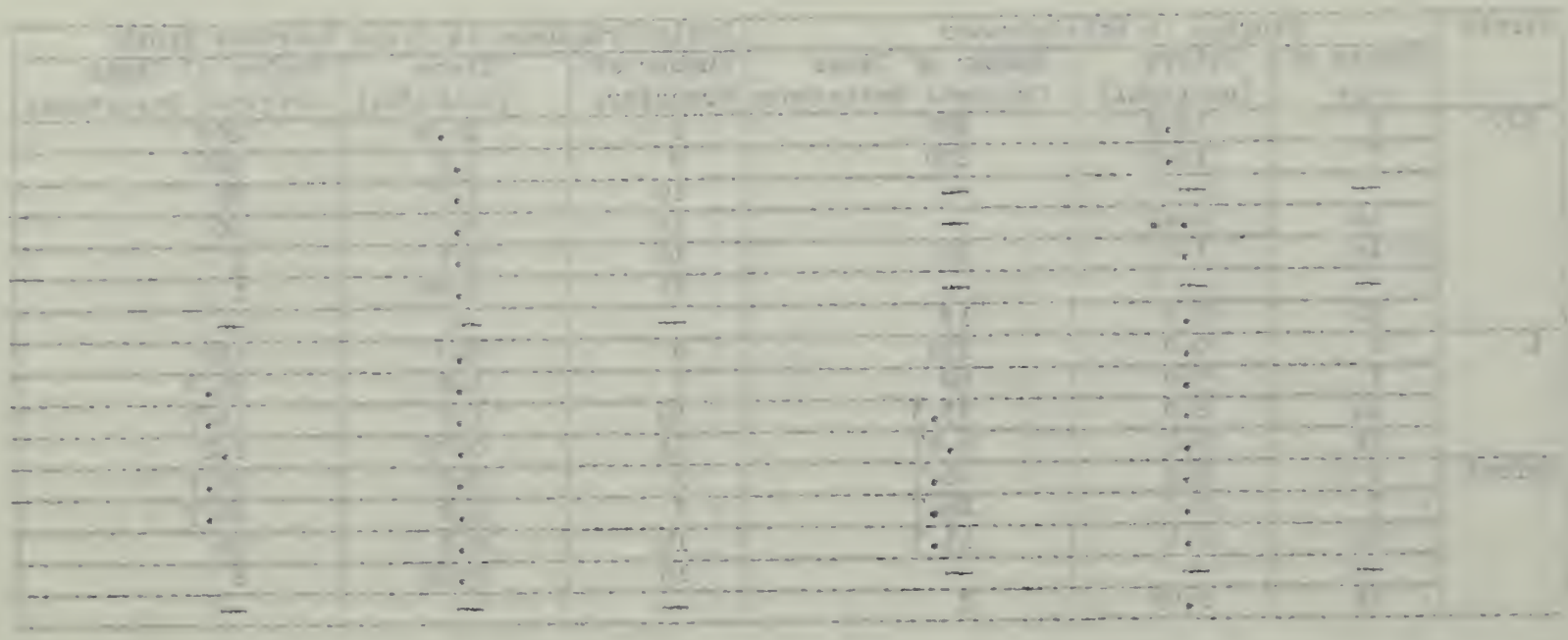
Strain H 109, beginning at 40 times the original resistance, remained at this figure after 29 days of storage. Strain 209 remained at 120 times its original resistance after 13 days in the refrigerator and dropped to only 100 times after 29 days of storage. Strain L again maintained its resistance with a drop from approximately 11 to 10 times the original resistance in 14 days, but showed a marked decrease to 3.3 times the original after 21 days of storage.

The results with penicillin were similar for decreasing resistance by daily transfers of the resistant cultures in penicillin-free broth; but there was a marked difference in the effects on storage in the refrigerator between penicillin and bacitracin. The penicillin-resistant cultures in all cases showed rapid drops in resistance both by storage in the refrigerator and by daily transfers in plain nutrient broth, although the latter was slightly more rapid. The results for decrease in resistance to penicillin are summarized in Table VII and Figures 10 A, B, and C. It can be seen that strain 209 dropped rapidly from 200 times to 2 times the original resistance after 16 transfers, and to its original resistance after only 21 transfers in plain broth. The stored culture dropped to 20 times the original after 16 days in the refrigerator. The Oxford

TABLE VII

Decrease in Resistance to Penicillin by 3 strains of Staphylococcus aureus

Strain	Storage in Refrigerator			Daily Transfers in Plain Nutrient Broth		
	Number of days	Titers (units/ml)	Number of Times Original Resistance	Number of Transfers	Titers (units/ml)	Number of Times Original Resistance
209	0	10.0	200	0	10.0	200
	7	10.0	200	8	5.0	100
	—	—	—	10	1.0	20
	14	n.s.	—	14	0.5	10
	16	1.0	20	16	0.1	2
	—	—	—	21	0.05	1
	30	0.9	18	—	—	—
L	0	9.0	150	0	9.0	150
	7	5.0	80	7	4.0	66.7
	14	2.0	33.3	14	1.0	16.7
	21	0.4	6.67	21	0.07	1.2
Oxford	0	2.0	66.7	0	2.0	66.7
	7	0.8	26.7	7	2.0	66.7
	14	0.4	13.3	14	0.3	10
	—	—	—	15	0.06	2
	21	0.06	2	—	—	—



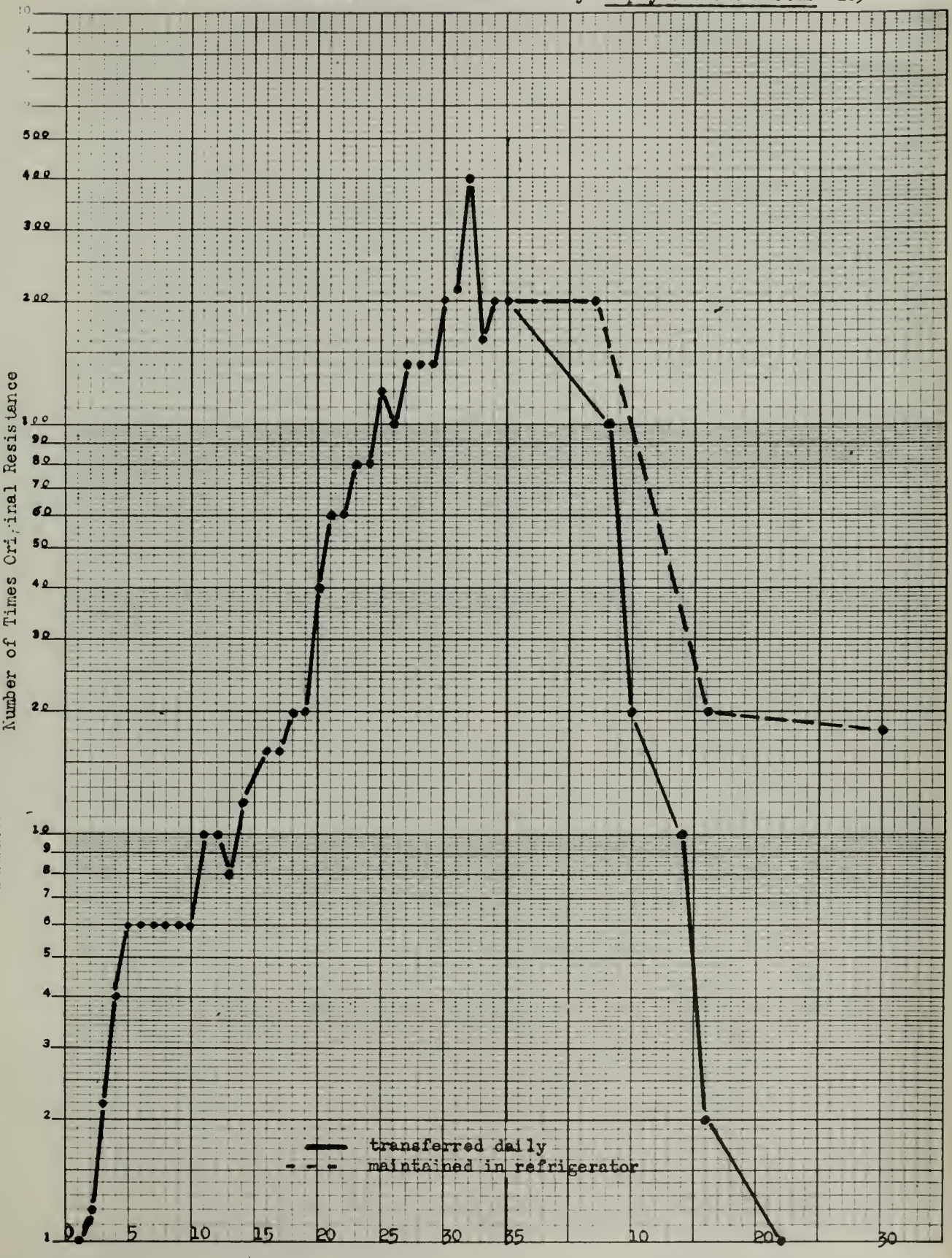

Number of Times Original Resistance
500
400
300
200
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0
5
10
15
20
25
30
35
10
20
30
transferred daily
maintained in refrigerator
Number of Exposures to Penicillin
Number of Days or Transfers in absence of penicillin

Figure 10 A

Rise and Fall in Resistance to Penicillin by Staphylococcus aureus Oxford

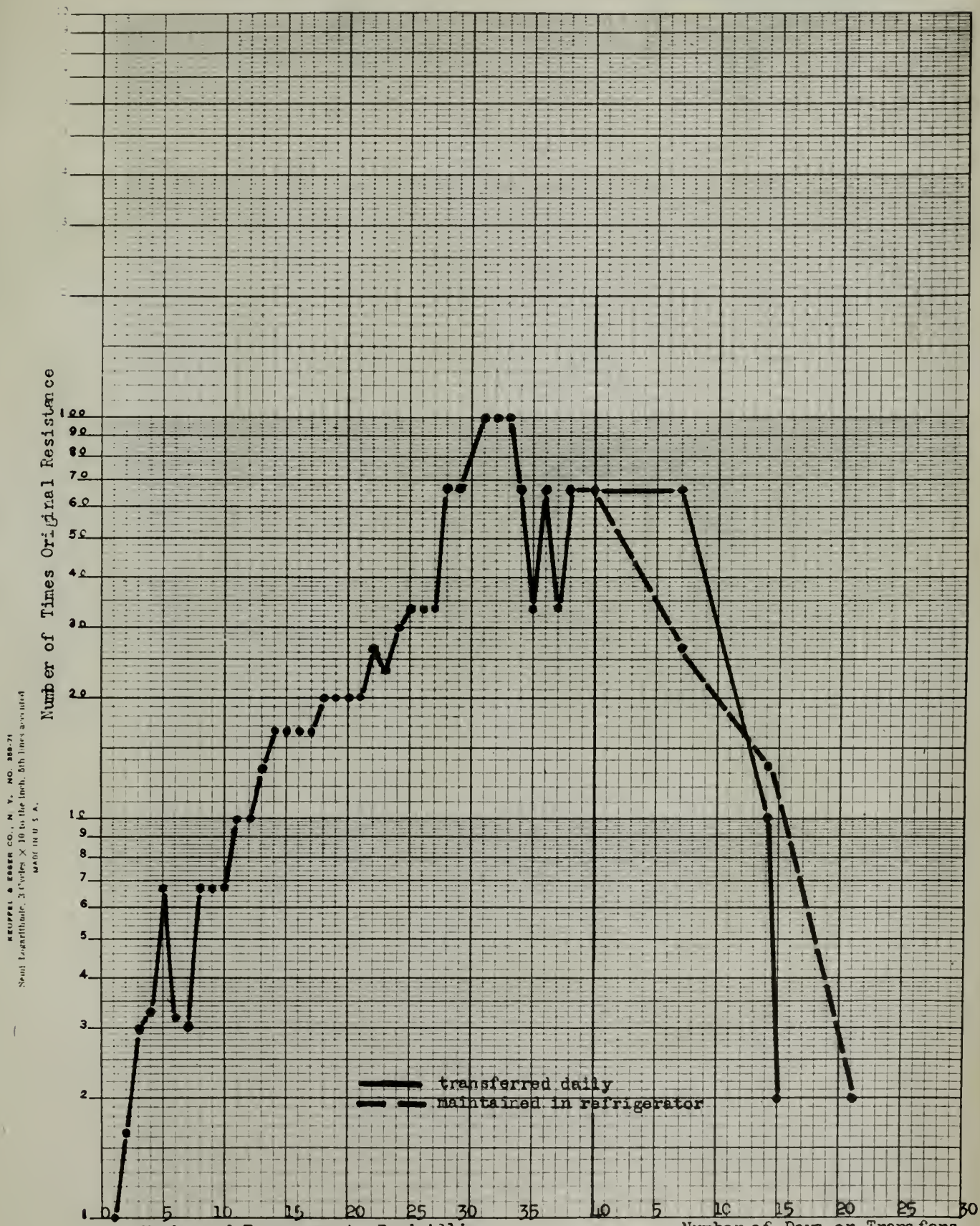


Figure 10 B

Rise and Fall in Resistance to Penicillin by Staphylococcus aureus Strain L

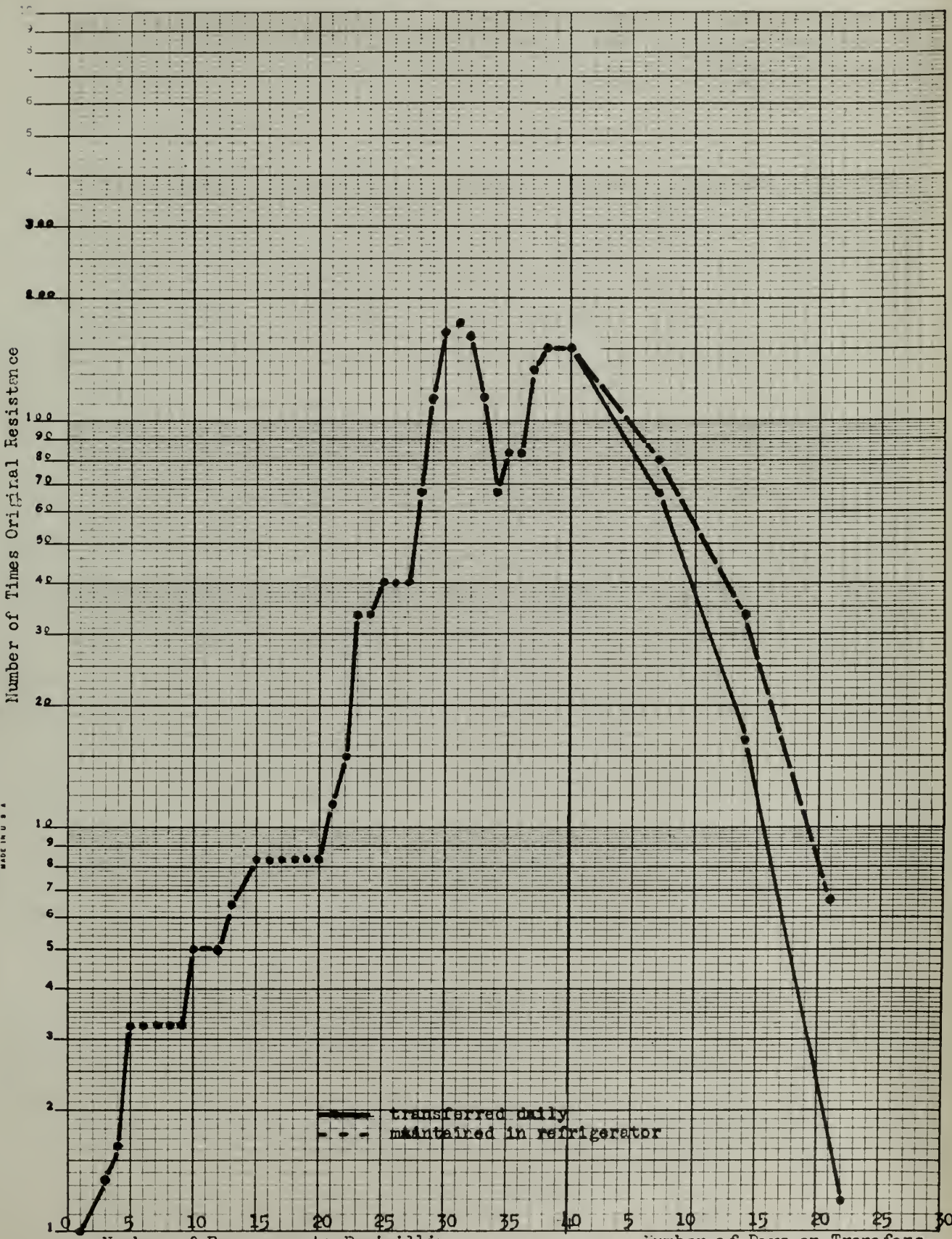


Figure 10 C

strain decreased its resistance from 66.7 times the original to 10 and 2 times the original in 14 and 15 daily transfers respectively, while the stored culture dropped to 13.3 times the original in 14 days of refrigeration. Strain L lost its resistance at a slightly slower rate than the other two, as the culture that was being transferred daily in nutrient broth dropped from 150 to 16.7 times its original resistance in 14 days, and the refrigerated culture dropped to 33.3 in 14 days.

D. Cross Resistance

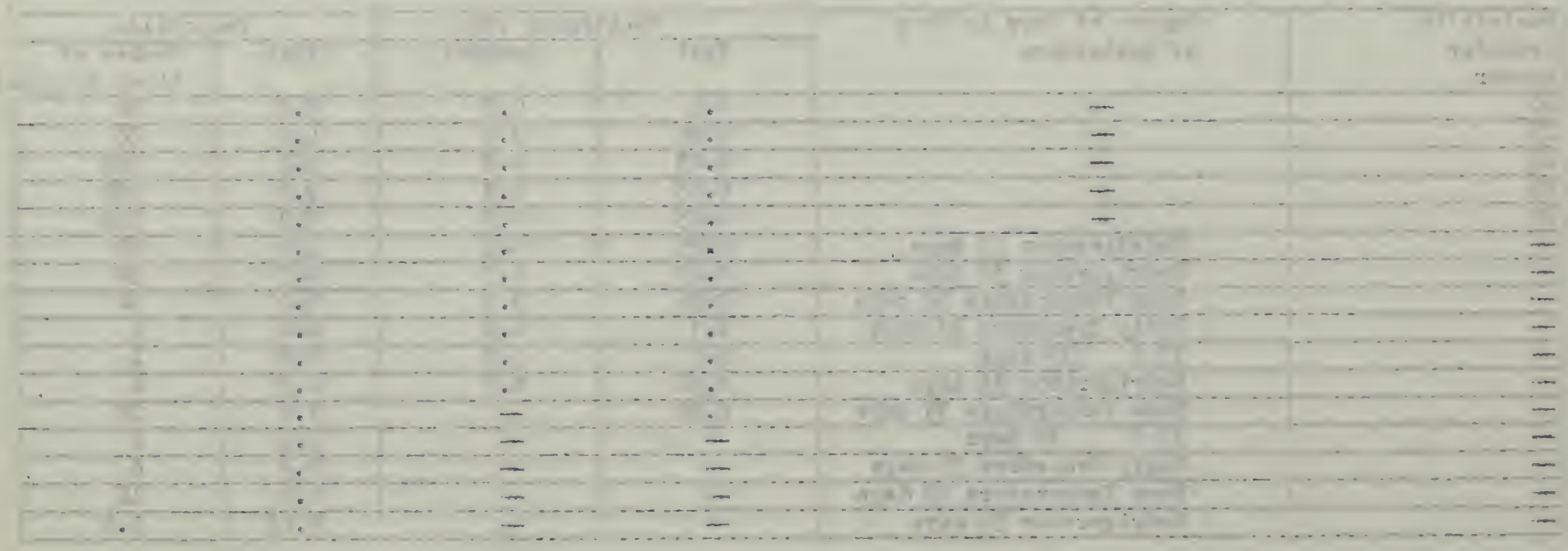
1. Preliminary Studies

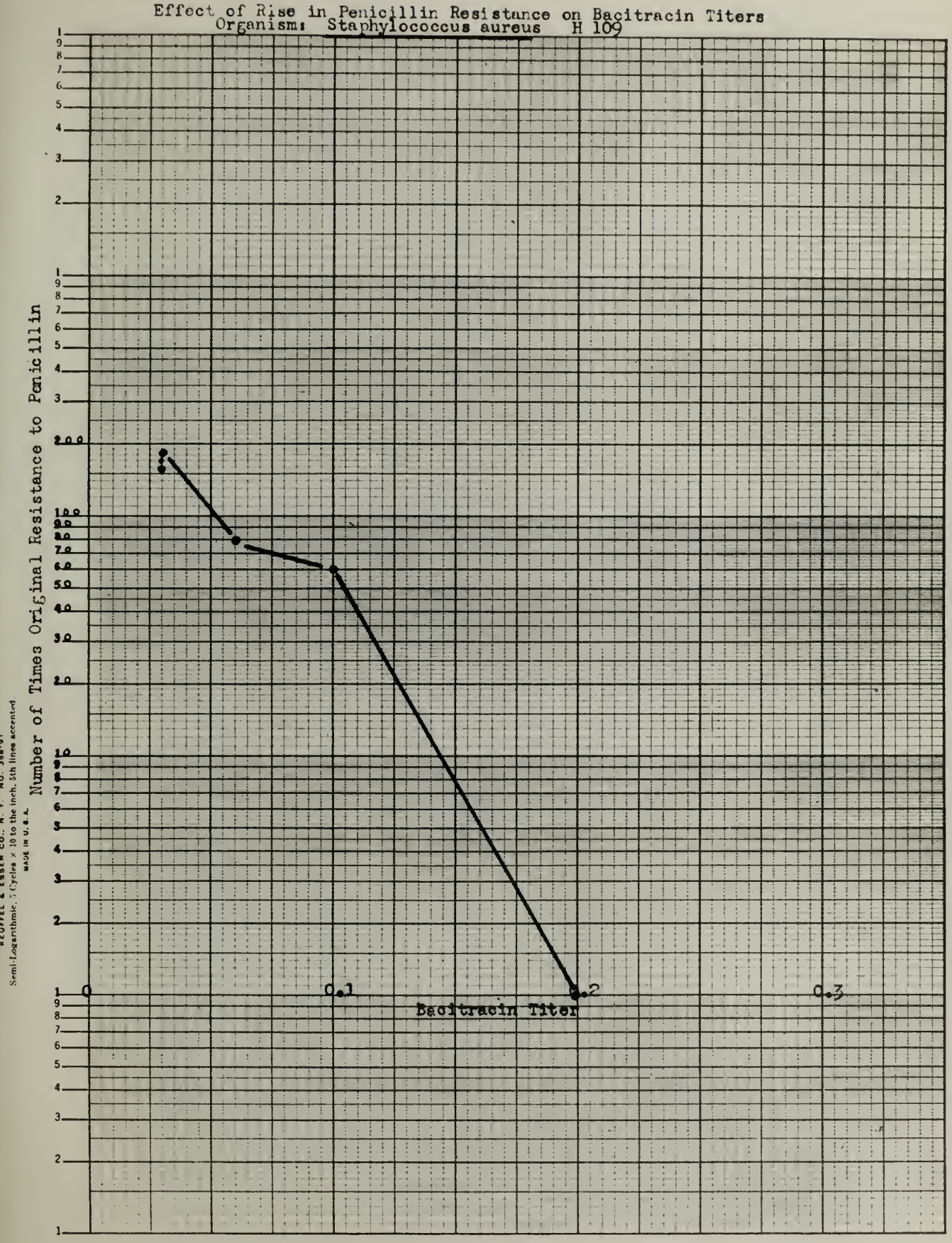
Penicillin alone was included in this series. The results of bacitracin titers accompanying the rise and fall of penicillin resistance is summarized in Table VIII and illustrated in Figures 11 A and B. There appears to be a decrease in bacitracin resistance accompanying the rise in penicillin resistance. The original end point of this strain for bacitracin was 0.2 units per ml of bacitracin. When the resistance to penicillin reached 80 times the original, the bacitracin titer dropped below 0.06 units per ml. At penicillin titers of 180 and 160 the bacitracin titer dropped to 0.03. With the drop of penicillin resistance, there was a rise of bacitracin resistance toward its original end point. There was no rise in bacitracin titer toward

TABLE VIII

Study of Cross Resistance to Bacitracin of Penicillin-resistant Staphylococcus aureus H 109

Penicillin Transfer Number	Number of Days in Drop of Resistance	Bacitracin Titer		Penicillin	
		Test	Control	Titer	Number of times original
10	—	0.1	0.3	3.0	60
15	—	0.06	0.3	4.0	80
18	—	0.03	0.2	9.0	180
19	—	0.03	0.3	9.0	180
20	—	0.03	0.3	8.0	160
—	Refrigerator 15 days	0.05	0.4	4.0	80
—	Refrigerator 32 days	0.03	0.3	0.5	10
—	Room Temperature 32 days	0.06	0.3	4.0	80
—	Daily Transfers 33 days	0.3	0.3	0.2	4
—	37 C 33 days	0.3	0.3	0.2	4
—	Refrigerator 33 days	0.02	0.3	0.3	6
—	Room Temperature 33 days	0.08	—	1.0	20
—	37 C 39 days	—	—	0.4	8
—	Daily Transfers 39 days	—	—	0.3	6
—	Room Temperature 39 days	—	—	0.6	12
—	Refrigerator 39 days	—	—	0.08	1.6




Effect of Rise in Penicillin Resistance on Bacitracin Titers
Organism: Staphylococcus aureus H 109
Number of Times Original Resistance to Penicillin
Bacitracin Titer
0
0.1
0.2
0.3
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Semi-Logarithmic, 5 Cycles × 10 to the inch, 5th lines accented
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Figure 11 A

normal by the resistant culture that was maintained in the refrigerator despite the drop in penicillin resistance.

2. Later Studies

As the resistance to bacitracin rose, there was no corresponding rise, but there appeared to be a slight drop in resistance to penicillin for 2 out of 3 strains, shown in Table IX. When the bacitracin resistance of strain H 209 was between 32 and 50 times its original resistance, the penicillin titers ran between 0.02 and 0.03 units per ml, while the control titers were in the range of 0.04 and 0.08 units per ml. For strain L the difference in penicillin titers was less marked since the test assays gave end points ranging from 0.05 to 0.07 units per ml, while the control showed end points of 0.07 and 0.08. There was, however, no indication of either increase or decrease of penicillin resistance accompanying increased resistance to bacitracin by strain 209. In a range of bacitracin resistance from 50 to over 125, all assays with penicillin gave end points of bacitracin resistant cultures that were identical with the control.

The penicillin resistant cultures gave evidence of being markedly altered in bacitracin susceptibility. The results of the assays of penicillin-resistant strains against bacitracin are summarized in Tables X, XI, XII, and XIII, and are represented graphi-

TABLE IX

Cross Resistance Studies of Bacitracin-resistant Staphylococcus aureus

Strains	Transfer Number	Bacitracin	Penicillin Titers (units/ml)	
		Number of Times Original Resistance	Test	Control *
H 109	48	32	0.02	not run
	49	50	0.02	0.06
	50	50	0.02	0.08
	54	50	0.02	0.07
	56	40	0.03	not run
	61	40	0.02	0.04
209	48	50	0.06	0.06
	50	90	0.06	0.06
	53	125	0.05	0.05
	61	100	0.06	0.06
L	23	8.33	0.06	0.08
	26	10	0.05	0.08
	34	8.33	0.07	0.07

* A 24 hour culture of the corresponding strain not exposed to either penicillin or bacitracin

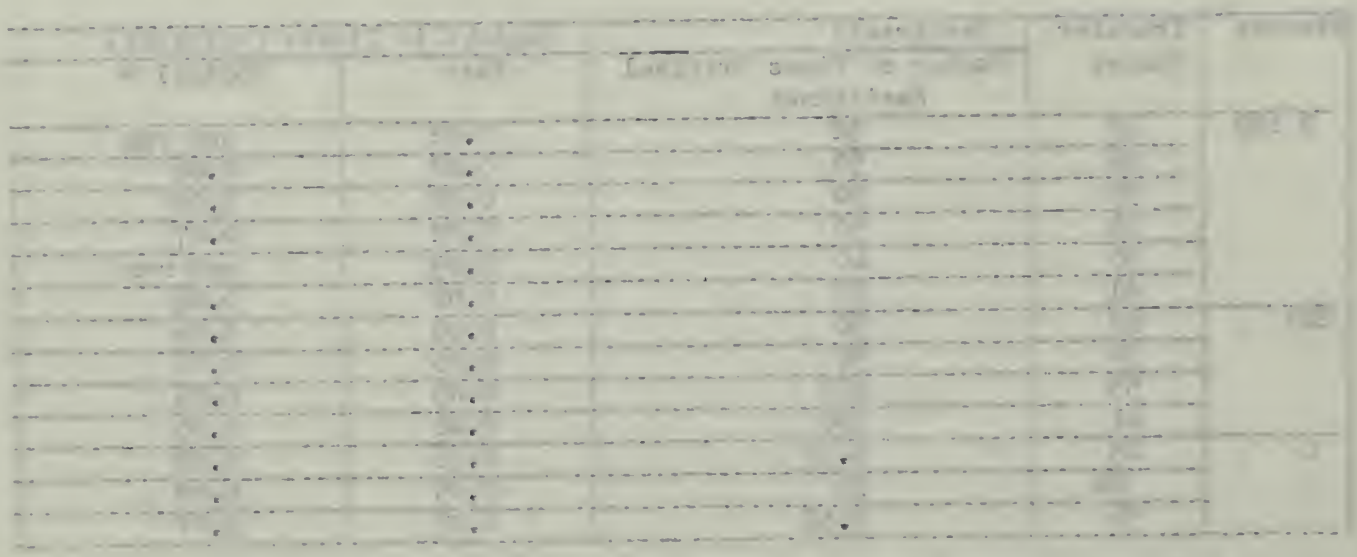


TABLE X

Cross Resistance Studies of Penicillin-resistant Staphylococcus aureus H 109

Transfer Number	Penicillin Number of Times Original Resistance	Bacitracin Titers (units per ml)	
		Test	Control
16	40	0.1	0.5
21	40	0.2	0.5
27	60	0.06	0.5
30	80	0.06	0.4
33	40	0.05	—
36	40	0.04	—

TABLE XI

Cross Resistant Studies of Penicillin-resistant Staphylococcus aureus 209

Bacitracin Titer (units/ml)		Penicillin		
Test	Control	Number of Times Original Resistance	Transfer Number in Rise of Resistance	Drop in Resistance
0.2	0.2	20	19	—
0.05	0.3	120	25	—
0.08	0.2	140	27	—
<0.01	0.3	140	29	—
0.06	0.2	400	32	—
<0.01	—	200	34	—
0.005	—	200	—	Refrigerator 8 days
0.07	—	20	—	Transfer Num ber 10
0.7	—	10	—	Transfer Number 14
0.6	—	1	—	Transfer Number 21

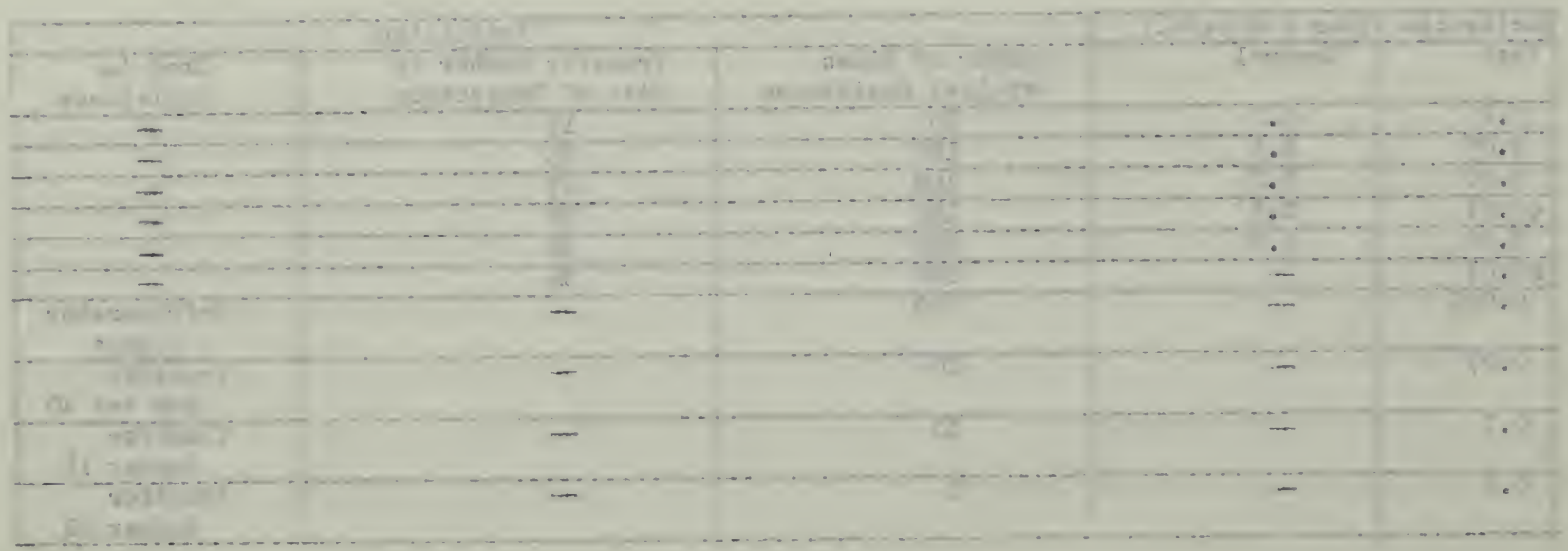


TABLE XII

Cross Resistance Studies of Penicillin-resistant Staphylococcus aureus Oxford

Bacitracin Titers (units per ml)		Penicillin		
Test	Control	Number of Times Original Resistance	Transfer Number in Rise of Resistance	Drop in Resistance
0.07	0.4	16.67	16	—
0.2	0.3	26.67	22	—
0.07	0.5	66.67	28	—
0.04	0.4	150	31	—
0.02	0.3	66.67	34	—
0.03	—	33.33	35	—
0.04	—	66.67	38	—
0.02	—	26.7	—	Refrigerator 7 days
0.04	—	66.7	—	Transfer Number 7
0.01	—	13.3	—	Refrigerator 14 days
0.2	—	1	—	Transfer Number 14
0.02	—	2	—	Refrigerator 21 days

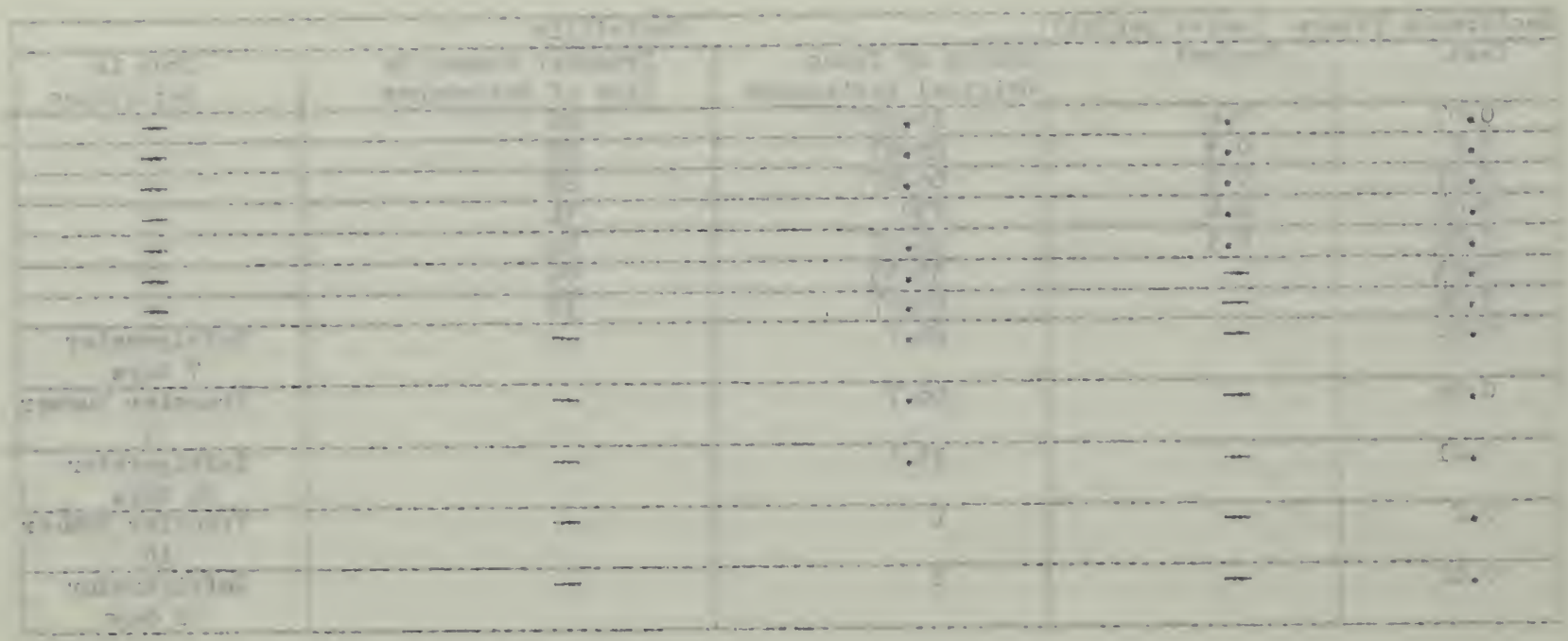


TABLE XIII

Cross Resistance Studies of Penicillin-resistant Staphylococcus aureus Strain L

Bacitracin titers (units/ml)		Number of Times Original Resistance	Penicillin Transfer Number in Rise of Resistance	Drop in Resistance
Test	Control			
0.06	0.3	8.33	18	—
0.06	0.5	33.33	24	—
0.03	0.3	50	27	—
0.02	0.5	116.67	29	—
<0.01	0.4	166.67	32	—
<0.01	0.6	150	38	—
0.02	—	80	—	Refrigerator 7 days
0.1	—	66.7	—	Transfer Number 7
<0.01	—	33.3	—	Refrigerator 14 days
0.5	—	16.7	—	Transfer Number 14
<0.01	—	6.67	—	Refrigerator 21 days
0.6	—	1.2	—	Transfer Number 21

Effect of Penicillin Resistance on Bacitracin Titers
Organism: Staphylococcus aureus 209

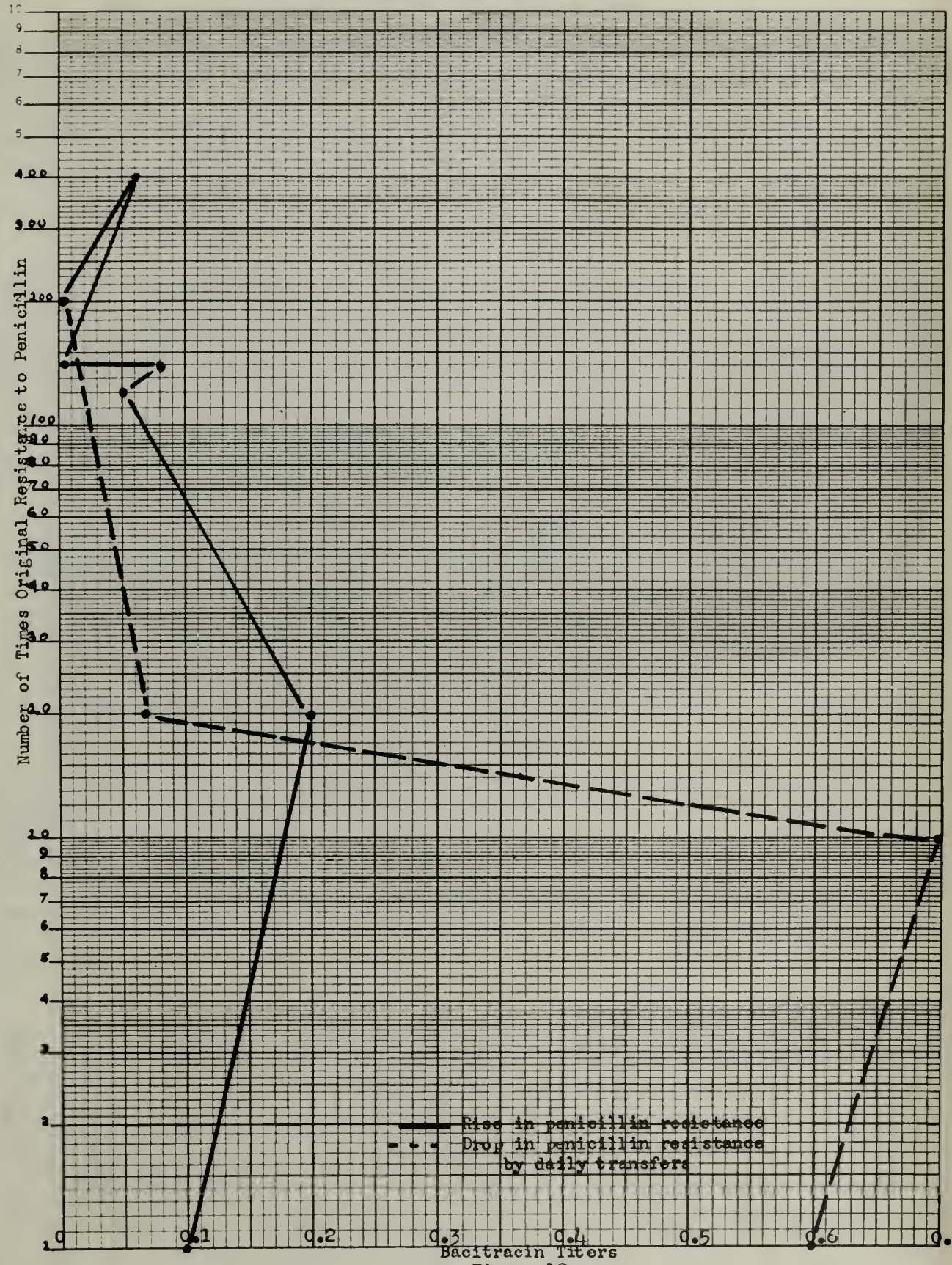
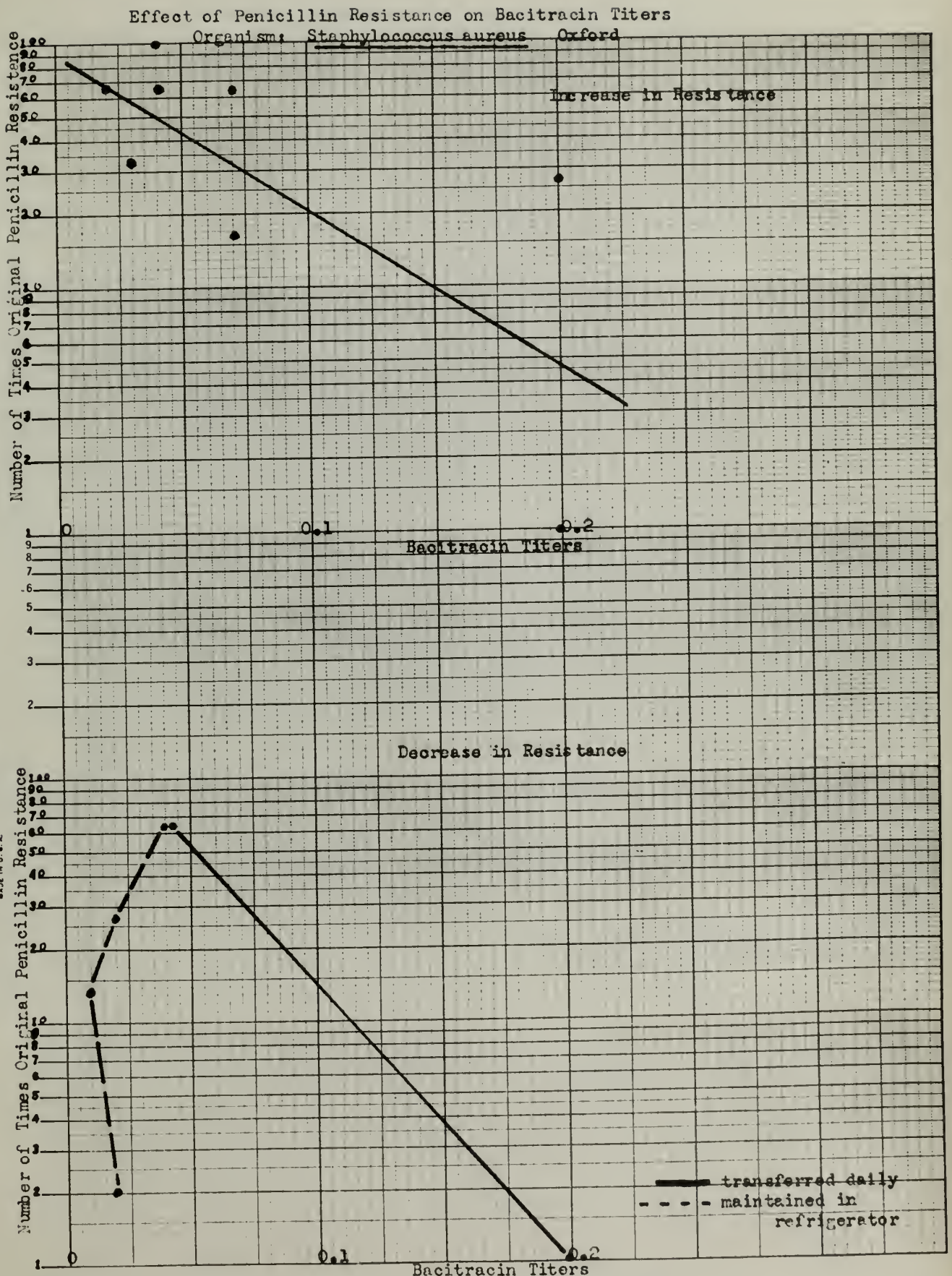


Figure 12



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Semi-Logarithmic, 3 Cycles x 10 to the inch, 5th lines accented
MADE IN U.S.A.

Figure 13

Effect of Penicillin Resistance on Bacitracin Titers

Organism: Staphylococcus aureus strain L

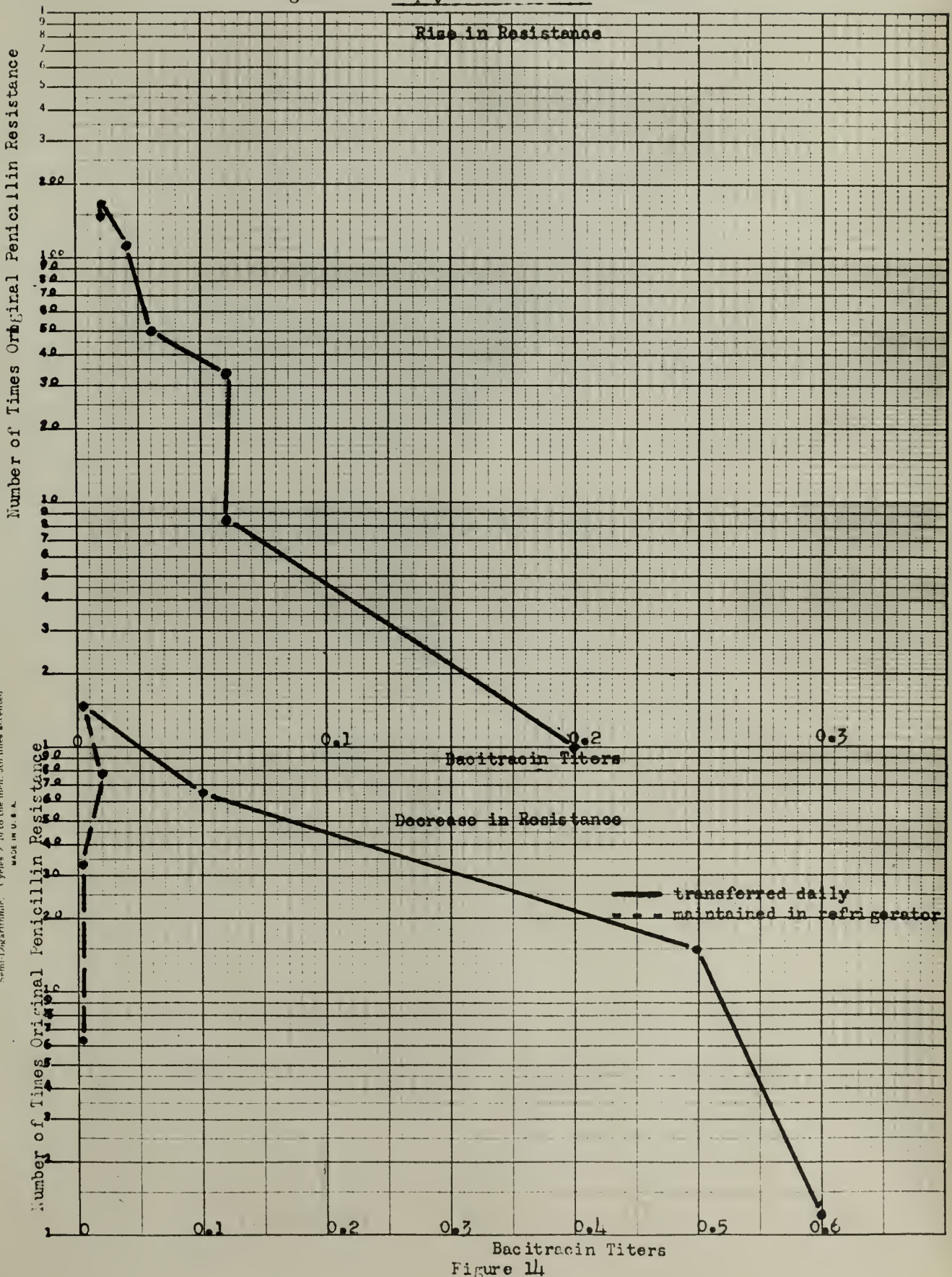


Figure 14

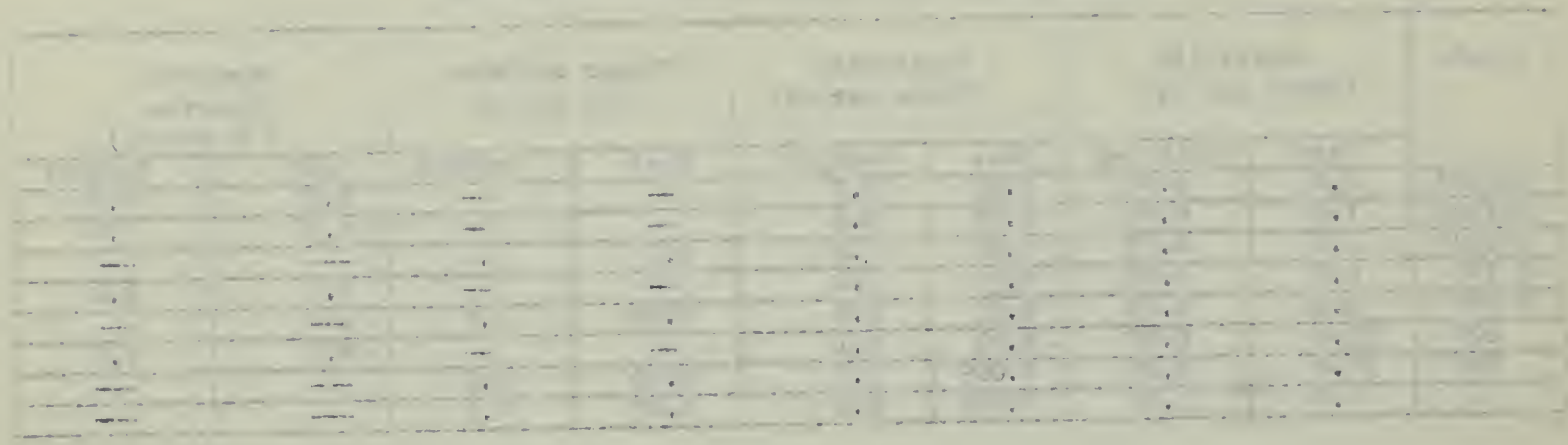
cally in Figures 12, 13, and 14. These tables and graphs confirm the preliminary studies by showing that as the penicillin resistance of a strain of Staphylococcus aureus increases, there is a simultaneous greater susceptibility to bacitracin. As the penicillin resistance returns toward normal, there is an concomitant return toward normal of the bacitracin titers with the exception of those cultures that have been refrigerated. The penicillin-resistant cultures that were maintained in the refrigerator lose their penicillin resistance nearly as rapidly as those that are transferred daily in plain nutrient broth, but the greater susceptibility to bacitracin appears to be more permanent since there is no accompanying return toward the normal titer.

The greater susceptibility to bacitracin of penicillin resistant cultures is not necessarily a specific phenomenon. Studies with other agents revealed that penicillin resistant strains became more susceptible to these agents in some instances. Table XIV shows that penicillin-resistant variants of strains H 109, L, and 209 became more susceptible to copper sulfate as well as bacitracin, and strain 209 increased its susceptibility to bichloride of mercury also. Strain H 109 did not alter its susceptibility to mercuric chloride, and the

Table XIV

Cross Resistance Studies
of
Three Agents with Penicillin Resistant Strains

Strain	Penicillin (Units per ml)		Bacitracin (Units per ml)		Copper sulfate (mg per ml)		Mercuric Chloride (γ per ml)	
	Test	Original	Test	Control	Test	Control	Test	Control
Oxford	2.0	0.03	0.02	0.3	—	—	0.8	1.0
Oxford	1.0	0.03	0.03	0.6	—	—	1.0	1.0
Oxford	2.0	0.03	0.04	0.2	0.2	0.2	—	—
H 109	2.0	0.05	0.05	0.2	—	—	1.0	1.0
H 109	2.0	0.05	0.04	0.2	0.06	0.3	—	—
209	10.0	0.05	0.01	0.1	—	—	0.5	1.0
209	10.0	0.05	0.005	0.1	0.03	0.2	—	—
L	8.0	0.06	0.009	0.2	0.07	0.2	—	—



Oxford strain changed its end point for neither bichloride of mercury nor copper sulfate, although its penicillin resistance rose and its bacitracin resistance dropped.

DISCUSSION

This study indicates that there is a relatively slow but steady rise of resistance to bacitracin by three out of four strains of Staphylococcus aureus, and the increase in resistance of these three follows a generally similar pattern. The fourth strain (the Oxford strain) gave no appreciable rise in resistance, the maximum attained during 35 exposures being 3-fold. Two strains (H 109 and 209) were carried to 63 and 62 exposures respectively, and attained maximum resistance levels of 80 and 125 respectively. These two strains at about the 25th or 30th exposure showed a tendency to level off in their rise of resistance at about 50 to 60 times the original resistances.

In contrast to the rise of resistance to bacitracin, the rise of resistance to penicillin was much more rapid and far greater. Strain 209 rose to a maximum of 400 times its original resistance in 32 exposures, strain H 109 rose to a maximum of 100 times in 32 transfers, and 200 times after 20 transfers in two separate runs, the Oxford strain and L strain reached peaks of 100 and 166.67 in 31 and 30 transfers respectively.

The decrease in resistance of strains that

became resistant by repeated exposure was also studied. Cultures that were made resistant to bacitracin would rapidly lose this resistance by transferring the cultures daily in plain nutrient broth so that in 29 days there was a gradual return nearly to normal. When, however, the bacitracin-resistant variants were maintained in broth at refrigerator temperatures, there was no appreciable loss of resistance in 2 out of 3 strains.

Penicillin showed a slightly different behavior in drop of resistance studies. All 4 strains that were studied showed a gradual decrease in resistance approaching the original level whether the cultures were refrigerated or transferred daily up to 21 days.

There was only a very slight tendency for bacitracin-resistant variants to affect the penicillin titers by resulting in a very little increase in susceptibility to penicillin. In contrast the penicillin-resistant cultures yielded a marked rise in susceptibility to bacitracin, so that strains which were originally inhibited at 0.2 units per ml of bacitracin would become altered so that less than 0.01 units per ml could prevent growth under otherwise identical conditions. This susceptibility of penicillin-fast strains to bacitracin became greater as the penicillin resistance rose,

and it decreased as the resistance to penicillin decreased with the exception of those cultures that were refrigerated. The penicillin-resistant variants, that were maintained in the refrigerator, lost their resistance to penicillin but maintained their increased susceptibility to bacitracin.

The increased susceptibility to bacitracin of penicillin-fast strains was not specific, as in some instances this increased susceptibility was also exhibited to other chemical agents, namely copper sulfate and bichloride of mercury. The reason for this increased susceptibility of resistant variants may be due to a decreased rate of growth, as suggested by Yegian, Budd, and Middlebrook.[80]

The parent cultures were assayed each day along with the test cultures as bacitracin controls. There were considerable variations in the end points of these controls with the same strain. The reason for this variation has not been determined. It was at first considered to be due to decreases in potency of the bacitracin, but continued study indicated that this was not a complete explanation, as using the same lot of bacitracin there would be a period at a certain level, followed by a rise in end point, and this followed by a subsequent return to normal. The possibility of some change in the culture was considered, but discarded, since it was unlikely that three or four

strains would simultaneously produce such a change. This explanation at first seemed logical, as it was previously shown that heavier inocula of the same strains yielded higher end points than the lighter inocula, and fluctuations in growth might produce cultures that were not constantly of the same density.

SUMMARY

1. The relative curve in rise of resistance by four strains of Staphylococcus aureus using penicillin and bacitracin as antibiotics was determined.
2. It was shown that penicillin developed resistance more rapidly and to a greater degree than did bacitracin.
3. There was a tendency for the rise of resistance to bacitracin to level off after 25 to 30 exposures at 50 to 90 times the original resistance.
4. Loss of resistance to penicillin developed rapidly by various methods of treatment, such as daily transfer in penicillin-free broth, storage in broth at room temperature, in the incubator, and in the refrigerator.
5. Loss of resistance to bacitracin was very rapid if the resistant cultures were transferred daily in bacitracin-free nutrient broth; but there was little or no decrease in bacitracin resistance following storage of the resistant cultures in the refrigerator up to 29 days.
6. Cross resistance studies with bacitracin-resistant cultures indicated that there was either no change in penicillin resistance or only a very slight decrease in the penicillin titers.

7. Cross resistance studies with penicillin-resistant strains indicated that as the resistance rose for penicillin, the organism became more susceptible to bacitracin. Conversely, as the resistance to penicillin decreased toward the original, the resistance to bacitracin rose toward its original point. This phenomenon was not entirely specific as in some instances the penicillin-resistant cultures also showed an increased susceptibility to mercuric chloride and copper sulfate.

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Induced Resistance to Bacitracin and Penicillin in Cultures of *Staphylococcus aureus*

Abstract of a Dissertation

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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by

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The purpose of this paper was to compare the rise in resistance curves by Staphylococcus aureus to penicillin and bacitracin, showing the relative rate and extent of drug fastness to these two antibiotics. The curves showing drop in resistance and the phenomenon of cross resistance were also to be investigated.

Many investigators have demonstrated that Staphylococcus aureus develops resistance rapidly to penicillin by repeated exposures to increasing concentrations of the drug. It has also been shown that microorganisms become resistant to many other antibiotics, notably streptomycin, both *in vitro* and *in vivo*. In general these induced resistances have been rapidly lost by transferring the cultures through a plain broth medium in the absence of the antibiotic. In some cases of streptomycin resistance, organisms have developed not only a permanent resistance but an actual dependence upon the drug as an essential or accessory growth factor.

Several antibiotic substances in addition to bacitracin have been isolated from strains of Bacillus subtilis. Bacitracin has been shown, however, to be one of the most promising of these antibiotics, and it has been one of the most extensively studied of this group. Studies on bacitracin have included methods of isolation and puri-

fication , some clinical evaluation, and various chemical and pharmacological properties including acute and chronic toxicity. The study of increases in resistance to bacitracin by susceptible organisms has, however, not as yet been reported/

Four strains of Staphylococcus aureus (H 109, FDA 209, L, and Oxford) were used for this study. The penicillin was commercial crystalline Penicillin G, and the bacitracin was a commercial product obtained through the courtesy of the Ben Venue Laboratories at Bedford, Ohio.

Titrations with both antibiotics were made using each strain to determine the initial end points of each organism to be studied. End point is defined as the least amount of antibiotic causing complete inhibition in growth of the test organisms.

Resistance was induced by transferring 0.5 ml of the end point tube to 10.0 ml of nutrient broth, and after incubation for 18 to 24 hours to obtain growth, the subculture was titrated to determine the new, higher end point. Repetitions of this procedure were employed to yield higher and higher end points.

Decreases in resistance were produced by maintaining a broth culture at refrigerator or other temperatures or by daily transfers in plain nutrient broth.

Cross resistance studies were made by determining the end point to one antibiotic of a culture resistant to the other antibiotic. For example, penicillin-resistant

cultures can be assayed with bacitracin in order to determine whether or not there is any increase or decrease in resistance to bacitracin as compared with the parent strain. Other unrelated chemicals, such as bichloride of mercury or copper sulfate can then be tested for this property in order to investigate the possibility that any variation might be non-specific.

This study indicates that there is a relatively slow but steady rise of resistance to bacitracin by three out of four strains of Staphylococcus aureus, and the increase in resistance of these three follows a generally similar pattern. The fourth strain (the Oxford strain) gave no appreciable rise in resistance, the maximum attained during 35 exposures being 3-fold. Two strains (H 109 and 209) were carried to 63 and 62 exposures respectively, and attained maximum resistance levels of 80 and 125 respectively. These two strains at about the 25th or 30th exposure showed a tendency to level off in their rise of resistance at about 50 to 60 times the original resistances.

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first seemed logical, as it was previously shown that heavier inocula of the same strains yielded higher end points than the lighter inocula, and fluctuations in growth might produce cultures that were not constantly of the same density.

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